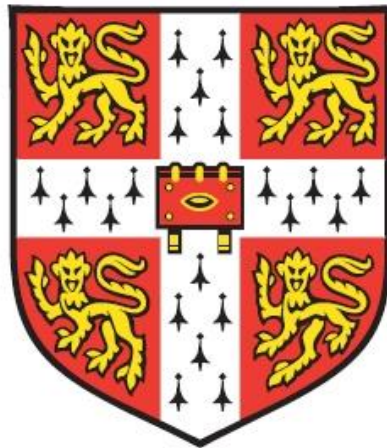

THE INFLUENZA A VIRUS NS1 PROTEIN AND VIRAL mRNA NUCLEAR EXPORT



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This dissertation is submitted for the degree of Doctor of Philosophy

Darwin College

University of Cambridge

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PREFACE

I hereby acknowledge that this thesis represents my own work and includes nothing that is the outcome of work done in collaboration except where specifically indicated in the text. The contents have not previously been submitted for a degree at this or any other university. This dissertation does not exceed the word limit length of 60000 words set by the degree committee.

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January 2018

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The submission of this thesis is the culmination of a lengthy and humbling journey where I truly understood the meaning of the word resilience. Life has no regards for our plans and, sometimes, reaching our goals will take following a more difficult and much, much longer path. I was, however, lucky to have met many incredible people over the years from whom I learned immensely and received invaluable support, for which I am grateful.

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The Influenza A Virus NS1 Protein and Viral mRNA Nuclear Export

SUMMARY

Influenza A virus (IAV) replication and transcription occur in the host cell nucleus; a feature which means both the viral genome (vRNA) and mRNA must be exported from the nucleus to the cytoplasm. The mechanism by which vRNA nuclear export is achieved has been well characterised, but how viral mRNAs are exported is poorly understood. The cellular NXF1-dependent mRNA export pathway has been shown to be involved in the export of some viral mRNAs, but how they are recruited to this pathway is unknown.

Prior work from our laboratory showed that segment 7 mRNA was inefficiently exported to the cytoplasm in a sub-viral ‘minireplicon’ system, providing the first indication that there were viral requirements for IAV mRNA nuclear export. Further addition of individual viral polypeptides was tested and the effect on segment 7 mRNA export was analysed by fluorescent *in situ* hybridization (FISH) and confocal microscopy. This identified the NS1 protein as the viral factor required for efficient segment 7 nuclear export.

Mutational studies on NS1 were carried out to unveil the mechanistic role of this protein in viral mRNA nuclear export, by plasmid transfection as well as in the context of recombinant viruses. These approaches indicated that both functional domains of NS1 were necessary to preserve the mRNA export function. Furthermore, these mutant proteins were used to examine the association between NS1 and the NXF1-dependent pathway in the context of mRNA nuclear export. Protein-protein and protein-RNA binding assays indicated that interactions between NXF1 and NS1, and NXF1 and segment 7 mRNA were necessary, but not sufficient to promote segment 7 viral mRNA export.

Lastly, the role of NS1 protein in the nuclear export of viral mRNAs from other genome segments was studied. The intracellular localisation of most viral mRNAs was not affected by the absence of NS1 or the presence of an export-incompetent NS1 mutant protein. However, segment 4 mRNA exhibited a similar phenotype to segment 7 mRNA in showing a dependence on NS1 for efficient nuclear export. Overall, the results presented in this dissertation suggest that NS1 acts as an adaptor protein between the viral RNA synthesis machinery and cellular export pathway. This provides deeper insights for the characterization of a recently identified function of the IAV NS1 protein, of being required for the efficient nuclear export of mRNA from “late” kinetic class viral genes.

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ABBREVIATIONS

3P	full heterotrimeric influenza polymerase complex
a.a.	amino acid
CHX	cycloheximide
CPSF	cleavage polyadenylation specificity factor
CRM1	chromosome region maintenance 1
cRNA	complementary RNA
DAPI	4,6-diamidino-2-phenylindole
dH ₂ O	deionised water
DMEM	Dulbecco's modification of Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribose nucleotide triphosphate
DRB	5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole
dsRNA	double-stranded RNA
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EJC	exon junction complex
EtOH	ethanol
FCS	foetal calf serum
Fig	figure
FISH	fluorescence in situ hybridization
GFP	green fluorescent protein
HA	haemagglutinin
HIV	human immunodeficiency virus
h	hour
hsp 70	heat shock protein 70
HSV	herpes simplex virus
IF	immunofluorescence
IFN	interferon
kDa	kilo Dalton
M1	matrix protein 1
M2	matrix protein 2

MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
NA	neuraminidase
NES	nuclear export signal
NLS	nuclear localization signal
NP	nucleoprotein
NPC	nuclear pore complex
NS1	non-structural protein 1
NS2/NEP	non-structural protein 2/nuclear export protein
NXF1	nuclear RNA export factor 1
p.i.	post-infection
p.t.	post-transfection
PA	polymerase acidic
PB1	polymerase basic 1
PB2	polymerase basic 2
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI3K	phosphoinositide 3-kinase
Pol I	DNA-dependent RNA polymerase I
Pol II	DNA-dependent RNA polymerase II
poly(A)	polyadenylic acid
PR8	influenza A/Puerto Rico/8/34
RdRp	RNA-dependent-RNA-polymerase
RFP	red fluorescent protein
RNAP II	DNA-dependent-RNA-polymerase II
Seg	segment
SFM	serum-free medium
snRNA	small nuclear RNA
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TREX	transcription/export complex
Udorn	influenza A/Udorn/72
UTR	untranslated region
vRNA	virion ribonucleic acid
vRNP	viral ribonucleoprotein complex
v/v	volume per volume

w/v	weight per volume
WB	western blot
WT	wild type

CHAPTER 1 – GENERAL INTRODUCTION

1.1 Influenza virus

The influenza A virus (IAV) belongs to the *Orthomyxoviridae* family, which also includes influenza B, C and D viruses, as well as Thogoto, infectious salmon anaemia (ISA) and Quaranja viruses. This virus family is characterized by having a genome organised as discrete segments of negative sense, single-stranded RNA (Palese and Shaw, 2007).

The host range of this family varies amongst its members. For example, Thogoto virus is a tick borne virus that infects vertebrates including humans while ISA is a salmon virus (Rimstad and Mjaaland, 2002). IAV is able to infect a wide range of mammalian and avian hosts while influenza B virus has so far only been detected in humans and seals and influenza C virus in humans and pigs (Guo et al., 1983). Recently, a new virus was discovered in cattle and pigs which was distant enough from influenza C to be proposed as influenza D virus, a new genus to the *Orthomyxoviridae* family (Hause et al., 2014; Hause et al., 2013). Even though the majority of influenza viruses are capable of infecting humans, IAV causes the most significant levels of serious and widespread disease, making it the major pathogen of the *Orthomyxoviridae* family.

IAV combines certain features that contribute to a high evolution rate. The viral polymerase is error-prone as it lacks a proof-reading ability, resulting in the accumulation of mutations which under selection give rise to antigenic drift. This equates to changes in the viral surface proteins that, in time, will be enough to make them unrecognisable by a pre-existing humoral immune response, thus effectively renewing the pool of susceptible hosts and resulting in seasonal epidemics. IAV is also capable of antigenic shift that can occur when different strains of virus infect a single cell, allowing the exchange of whole genome segments during the formation of new virus particles (Palese and Shaw, 2007). This phenomenon can result in the emergence of a new virus subtype, for which there is no widespread immunity, possibly resulting in a pandemic. Pandemics can potentially also occur upon adaptation of completely avian viruses to human hosts (Taubenberger et al., 2005). IAV caused several pandemics throughout the 20th century, the most notorious being the “Spanish” 1918 pandemic which has been estimated to have infected one third of the population at the time (~500 million people) and to have been fatal to ~50 million people worldwide (Taubenberger and Morens, 2006). The only pandemic (so far) of the 21st century involved a new variant of H1N1 influenza virus; “swine flu” occurring in 2009. This new strain of the

virus appears relatively mild in terms of pathogenicity, but is highly transmissible (Peiris et al., 2009; Sullivan et al., 2010). Although the World Health Organisation (WHO) confirmed 18,500 deaths during the pandemic phase, it has been estimated that “swine flu” was responsible for 284,500 mortalities (Dawood et al., 2012).

The evolutionary potential that influenza A virus has due to its rapid ability to change together with its vast host range, keeps the threat of new pandemics resulting from highly pathogenic new strains alive.

1.2 Influenza A virus proteins

The eight segments of influenza A virus have been shown to encode at least 17 polypeptides. Each segment encodes at least one essential “core” polypeptide (segments 7 and 8 encode two) but most other segments can produce alternative non-essential polypeptides, often in a virus strain-dependent manner. Segment 1 encodes PB2 (polymerase basic 2) which is part of the viral polymerase heterotrimer and has pre-mRNA cap binding activity (Li et al., 2001; Plotch et al., 1981). PB2 has also been shown to be imported to mitochondria and to associate with the mitochondrial antiviral signalling (MAVS) protein resulting in the inhibition of beta interferon, thereby affecting the virus virulence (Graef et al., 2010; Long and Fodor, 2016). Some strains of IAV additionally produce a minor PB2-related polypeptide, PB2-S1, of unknown function from a spliced transcript (Yamayoshi et al., 2015). Segment 2 primarily codes for PB1 (polymerase basic 1) which is a second constituent of the trimeric viral polymerase and the one that contains the RNA polymerase catalytic domain (Biswas and Nayak, 1994; Braam et al., 1983). A truncated form of PB1 is also encoded by this segment, producing the PB1-N40 protein. This protein has been shown to interact with the polymerase complex in the cell nucleus, although its function remains unknown (Wise et al., 2009). A second overlapping open reading frame in segment 2 encodes a third protein, PB1-F2, which has been associated with a variety of roles including pathogenesis, apoptosis and transmission (Chen et al., 2001; Conenello and Palese, 2007; James et al., 2016). Segment 3 encodes the final protein part of the polymerase complex; PA (polymerase acidic) and contains both RNA endonuclease and suggested protease activities (Hara et al., 2001; Sanz-Ezquerro et al., 1996; Yuan et al., 2009; Zhao et al., 2009). Truncated forms of PA have been identified, PA-N155 and PA-N182 proteins, whose functions are unknown, but have been shown to lack polymerase activity (Muramoto et al., 2013). This segment also encodes the PA-X protein, produced via a +1 ribosomal frameshifting event, which has been shown to modulate the host response to IAV infection *in vivo* (Jagger et al., 2012).

Segment 4 encodes the viral transmembrane surface glycoprotein haemagglutinin (HA), which is responsible for binding sialic acid residues on host cell receptors as well as inducing fusion of the viral envelope and endosomal membrane during entry (Skehel and Wiley, 2000). Segment 5 encodes NP (nucleoprotein), whose main function is to encapsidate vRNA and cRNA (Shapiro and Krug, 1988) and which is also required for viral genome transcription and replication (Honda et al., 1988; Huang et al., 1990). Segment 6 encodes the viral transmembrane surface glycoprotein neuraminidase (NA), which is responsible for digesting sialic acid residues on host cell surface allowing the release of viral particles from the infected cell (Palese et al., 1974).

Segment 7 encodes primarily two proteins produced from two different species of the same mRNA; an unspliced form that encodes M1 (matrix 1) and a spliced form which encodes M2 (matrix 2). M1 protein is a major virion structural protein that links vRNPs to the viral envelope. This protein is also responsible for vRNP nuclear export upon binding firstly NP protein and secondly NS2 (non-structural 2) (Martin and Helenius, 1991; O'Neill et al., 1998). M2 protein is a membrane protein that acts as an ion channel in the viral envelope (Pinto et al., 1992), allowing the acidification of the virus particle interior necessary for uncoating (Bukrinskaya et al., 1982; Hay et al., 1985). A third protein, M42, can also be encoded by segment 7. This protein is M2-related, carrying an antigenically different ectodomain and has been shown to be a functional substitute for M2 protein (Wise et al., 2012).

Segment 8 also produces different mRNA species resulting from splicing. The unspliced mRNA species encodes NS1 (non-structural 1) to which numerous functions have been attributed, the main one being an interferon antagonist (Hale et al., 2008b). NS2 (also known as NEP, for nuclear export protein) is encoded by the spliced mRNA species and is responsible for vRNPs nuclear export upon M1 binding as mentioned above (Neumann et al., 2000; O'Neill et al., 1998). Further proteins encoded by segment 8 have been recently identified. These include NS3 (non-structural 3), which is an alternatively spliced variant of NS1 that has been linked to host switching (Selman et al., 2012) and more recently, N-terminally deleted versions (tNS1; starting at codons 79 and/or 81) which may play a role in inhibiting IRF3 (Kuo et al., 2016).

1.3 Influenza A virus structure

Influenza A virions range from 60-100 nm in diameter to more than 10 μm in length depending on whether their shape is spherical or filamentous (Bourmakina and Garcia-Sastre, 2003; Bruce et al., 2010; Dadonaite et al., 2016). Each particle (shown in a diagrammatic representation in **Figure 1.1**) is enveloped with a lipid membrane acquired from the host cell plasma membrane upon budding and release. The viral glycoproteins HA and NA are present on the particle surface (Fujiyoshi et al., 1994; Harris et al., 2006; Yamaguchi et al., 2008) and the viral ion-channel protein M2 is also incorporated into the envelope (Pinto et al., 1992).

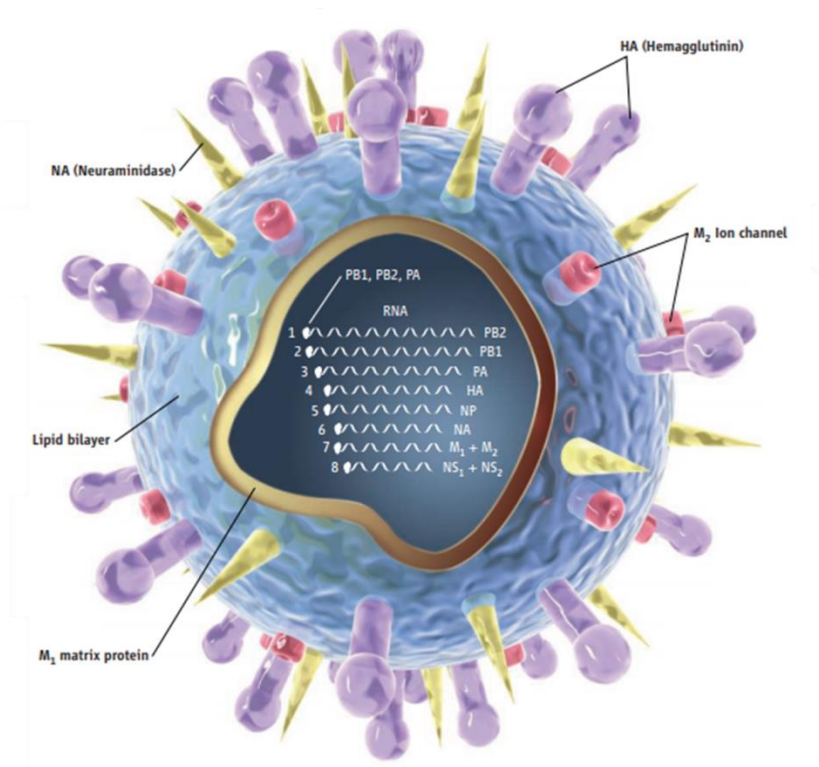


Figure 1.1 The IAV particle. In the core of the virion the segmented viral genome is packaged into vRNPs, each RNA being encapsidated with multiple copies of NP and attached to a trimeric polymerase consisting of PB2, PB1 and PA. The viral protein M1 links the vRNPs to the lipid envelope where the M2 ion channel and the glycoproteins HA and NA sit. Taken from (Kaiser, 2006)

The interior of the virion contains one copy of each of the eight negative sense, single strands of the RNA viral genome (Hutchinson et al., 2010). Every segment is encapsidated with multiple copies of NP protein, each monomer covering 20-24 vRNA nucleotides (Compans et al., 1972). Furthermore, each individual segment is associated with one copy of the viral encoded polymerase complex consisting of PB2, PB1 and PA proteins. The viral RNA together with NP protein and the trimeric viral polymerase form a viral ribonucleoprotein (vRNP). All eight IAV segments are enclosed by the viral protein M1

which is positioned beneath the envelope linking it to the vRNPs. The viral NS2 protein and, more recently NS1 protein, have also been shown to be incorporated in low copy number into the virion (Hutchinson et al., 2014; Richardson and Akkina, 1991).

1.4 Influenza A virus life cycle

The IAV virus life cycle (a representation of which is shown in **Figure 1.2**) is relatively short (8-10 h in cell culture) and starts with the entry of the virus into the target cell, followed by its uncoating. Transcription and replication of the viral genome take place in the cell nucleus, which is a relatively uncommon feature for an RNA virus. Expression of viral proteins is followed by assembly of new virions and the life cycle ends with the release of the newly produced virus particles from the host cell.

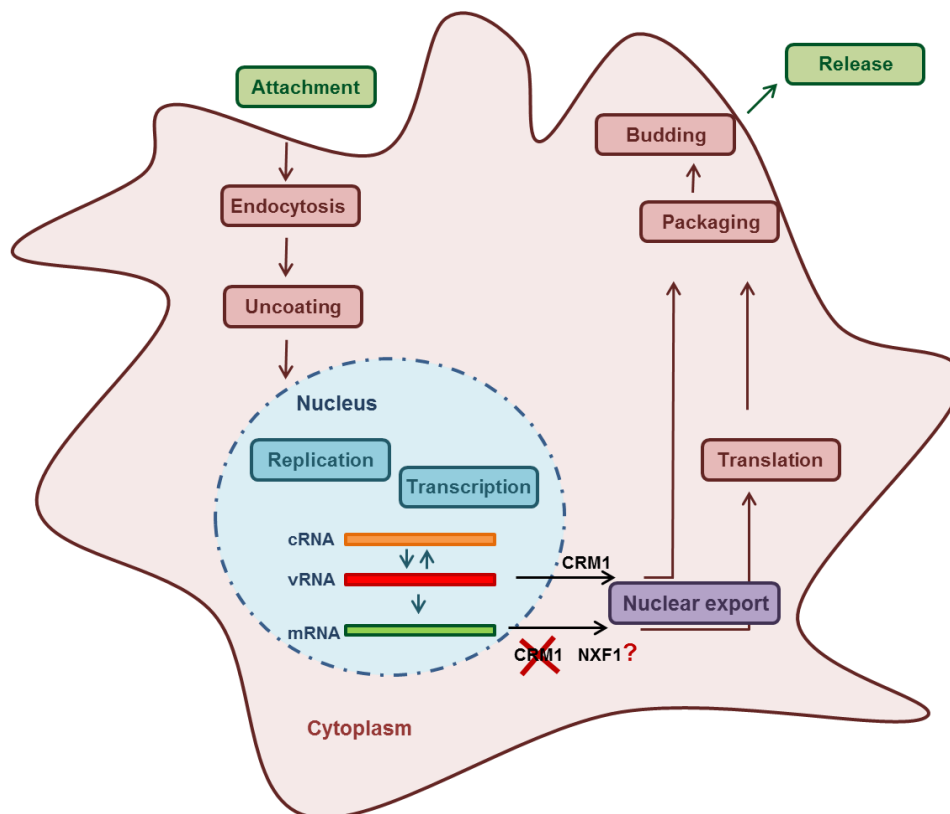


Figure 1.2 The IAV life cycle. Infection is initiated with the attachment of HA to sialic acid residues present on the surface of the host cell, triggering endocytosis. Acidification of the endosome results in fusion of viral and endosomal membranes, as well as the uncoating and release of the vRNPs into the cytoplasm which are imported to the cell nucleus. Transcription occurs in the nucleus and mRNAs are subsequently exported to the cytoplasm where they are translated into new viral proteins. Replication of the genome occurs via the production of an intermediate positive-sense cRNA which is used as a template for the synthesis of new, full-length, negative sense vRNA molecules which are packaged with NP protein. The vRNPs are then exported to the cytoplasm and trafficked to the apical cell surface where a single copy of each of the eight segments is packaged into a new virion which buds from the cell surface and is thereby released from the host cell.

1.4.1 Viral entry into the host cell

IAV entry is achieved by the binding of the viral glycoprotein HA to sialic residues present on the surface of target cells. This interaction triggers receptor-mediated endocytosis, resulting in the internalisation of the virus particle (Gottschalk, 1959; Marsh and Helenius, 2006; Matlin et al., 1981; Rust et al., 2004; Sieczkarski and Whittaker, 2005). The acidification of the endosomes carrying virus particles stimulates a conformational change of HA, exposing a fusion peptide which upon insertion into the endosomal membrane leads to its fusion with the viral envelope (Skehel and Wiley, 2000). Endosome acidification also results in the influx of protons into the viral particle through the viral ion channel M2 (Pinto et al., 1992). The pH drop inside the viral particle results in the dissociation of M1 and vRNPs, making them competent for nuclear import upon release into the cell cytoplasm (Martin and Helenius, 1991).

Viral RNP size determines that nuclear import must occur via active processes. Nuclear localisation signals (NLS) present on NP protein are thought to mediate vRNP import into the nucleus by interacting with the importin alpha/beta pathway and nuclear pore complex components (Cros et al., 2005; Cros and Palese, 2003; O'Neill et al., 1998; Wu and Pante, 2009).

1.4.2 Genome transcription

Transcription of the viral genome takes place in the host cell nucleus and starts once vRNPs are imported to this compartment from the cytoplasm (Herz et al., 1981). The viral mRNAs resemble cellular ones and possess 5'-cap structures and 3'-poly(A) tails (York and Fodor, 2013).

Contrary to the non-segmented, negative-strand RNA viruses, the IAV polymerase does not carry methyltransferase activity, which means that it is incapable of 5' capping viral RNA transcripts (Plotch et al., 1979). Instead, IAV utilises host mRNAs as a source for capped primers in order to initiate viral transcription via a 'cap-snatching' mechanism (Plotch et al., 1981). The viral polymerase complex binds both ends of the viral genome that, annealed into a panhandle structure, form the promoter for viral transcription initiation (Fodor et al., 1994; Hagen et al., 1994; Tiley et al., 1994). This activates the cap-binding activity of the viral polymerase subunit PB2 which interacts with the 5' m⁷G-caps of cellular pre-mRNAs (Blaas et al., 1982; Braam et al., 1983; Fechter et al., 2003; Guilligay et al., 2008). Host encoded pre-mRNAs are then cleaved 10-13 nucleotides downstream of the cap structure by PA which possesses RNA endonuclease activity (Crepin et al., 2010; Dias et al.,

2009; Hara et al., 2001; Plotch et al., 1981; Yuan et al., 2009). The transcription reaction is catalysed by PB1 upon binding capped cellular pre-mRNA fragments which are used as primers for synthesis of the viral transcripts (Cianci et al., 1995; Li et al., 1998a; Plotch et al., 1981; Ulmanen et al., 1981). This intricate set of interactions and enzymatic reactions has long been suspected to involve a series of conformational changes in the polymerase trimer. This supposition has recently been much strengthened by a series of crystallographic structures of the IAV as well as influenza B and C virus polymerase complexes which provide direct evidence of P protein domain movements between different activity states (Hengrung et al., 2015; Pflug et al., 2014; Reich et al., 2014).

Viral transcripts are polyadenylated at the 3' end as are host mRNAs; however, the 3' end processing of both types of pre-mRNAs is achieved via distinct mechanisms. Host pre-mRNA polyadenylation occurs using the cellular polyadenylation machinery. Eukaryotic cellular mRNAs contain a conserved signal sequence (AAUAAA) preceding poly(A) sites that is recognised by the cleavage polyadenylation specificity factor complex (CPSF), which is required for both the cleavage and polyadenylation reactions (Zhao et al., 1999). Following endonucleolytic cleavage, addition of adenosine residues to the mRNA is catalysed by a poly(A) polymerase (PAP), although the rapid elongation and control of the poly(A) tail length require the participation of poly(A)-binding protein II (PAB II). PAB II also stabilises the polyadenylation complex via its interaction with CPSF-30, a 30- kDa subunit of CPSF (Zhao et al., 1999). In contrast, IAV transcripts acquire poly(A) tails via the stuttering of the viral polymerase on a poly(U) stretch at the 5' end of the vRNA (Robertson et al., 1981). The poly(U) stretch at the 5' end of the viral segments has been shown to be required for mRNA synthesis and moving it resulted in loss of function of the motif (Luo et al., 1991). Replacement of uridines to adenines in the template vRNA resulted in poly-uridylated mRNAs, strongly supporting a model proposing that as mRNAs are being synthesized, the loop of transcribed vRNA gets shorter, eventually leading to the polymerase stuttering at the 5' end of the vRNA where the uridine motif is located because of steric hindrance (Poon et al., 2000; Poon et al., 1998). Furthermore, whereas vRNPs have been shown to be able to replicate in *trans*, transcription only occurs in *cis*, suggesting that the viral polymerase is required to remain bound to the 5' end of the template for the stuttering on the poly(U) stretch to occur (Jorba et al., 2009).

IAV produces three different classes of mRNA which can be intronless (in most IAV strains, segments 1-6), spliced or unspliced (segments 7 and 8). Viral transcripts can also be categorised according to the kinetics of their gene product's expression during infection. Segments, 1-3, 5 and the unspliced segment 8 fall into the 'early' category while segments 4, 6, 7 and spliced segment 8 would be classified as 'late' gene mRNAs (Hatada et al., 1989; Inglis and Mahy, 1979; Lamb et al., 1978; Skehel, 1972).

While it has long been accepted that IAV uses the cell host machinery for the splicing of its mRNAs (Lamb and Horvath, 1991), the regulation of this process remains to be fully elucidated.

Segment 8 can give rise to NS1 or NS2 proteins depending on whether the unspliced or spliced transcripts are synthesised, respectively (Inglis et al., 1979; Lamb et al., 1980; Lamb and Lai, 1980). More recently, a novel protein - NS3 - has been shown to result from alternative splicing of segment 8 mRNA through the mutational activation of a different splice donor site. All strains of IAV are expected to make NS1 and NS2, while NS3 is a rare variant. Splicing of segment 8 mRNA has been proposed to be regulated by viral factors which include NS1 (Chua et al., 2013; Garaigorta and Ortin, 2007; Smith and Inglis, 1985). Furthermore, the nuclear export of unspliced NS1 mRNA was suggested to play a role in regulating the extent of NS1 mRNA splicing (Alonso-Caplen and Krug, 1991). However, other studies have found NS1 not to alter the levels of NS2 spliced transcript accumulation while reducing the levels of spliced cellular mRNAs, suggesting that splicing of IAV segment 8 is independent of NS1 (Robb et al., 2010). This study is in agreement to a previous one that suggested that while NS1 inhibited splicing of cellular mRNAs, NS1 mRNA itself was resistant to this inhibition (Lu et al., 1994). More recently, an exonic splicing enhancer motif (ESE) has been identified in the NS2 mRNA which was shown to interact with host splicing regulator SF2 and thereby regulate segment 8 mRNA splicing (Huang et al., 2017). IAV has also been proposed to use suboptimal splicing (Lamb and Lai, 1984) as a strategy to coordinate the timing of infection, ensuring the slow accumulation of NS2 while high levels of NS1 are produced (Chua et al., 2013).

Segment 7 on the other hand has been shown to produce up to four different transcripts (**Figure 1.3**) and will be described in greater detail as this dissertation focuses mainly on the nuclear export of the unspliced mRNA produced from this segment (Inglis and Brown, 1981; Lamb and Choppin, 1981; Lamb et al., 1981; Shih et al., 1998; Wise et al., 2012). The unspliced mRNA1 encodes M1 protein, while spliced mRNA2 encodes M2 protein. mRNA4 has been shown to encode M42, an M2-related protein that can functionally replace M2 (Wise et al., 2012). This mRNA has also been hypothesised to encode another

protein, M4, although this polypeptide has not yet been detected (Shih et al., 1998). The remaining spliced transcript, mRNA3, is thought to play a role in regulating the levels of segment 7 spliced products. In particular, the blockage of mRNA3 5' splice site by the viral polymerase binding to the 5'-promoter sequence was shown to result in preferential usage of the mRNA2 5' splice site, enabling the production of mRNA2 (Shih et al., 1995). Although mRNA3 has the coding potential for a 9 amino acid peptide, this remains to be identified (Lamb et al., 1981). Segment 7 spliced mRNAs have one feature in common in that they use the same 3' splice acceptor site and each uses a specific 5' donor splice site (**Figure 1.3**). Similarly to splicing of segment 8, most if not all IAV strains will produce mRNAs 1 and 2, whereas the production of mRNAs 3 and especially 4 are more strain-specific (Shih et al., 1998; Wise et al., 2012).

The regulation of splicing events in segment 7 transcripts has been studied extensively over decades and yet, much is still unknown about how IAV ensures that each mRNA is produced in the required amounts at different times of infection. In addition to the potential

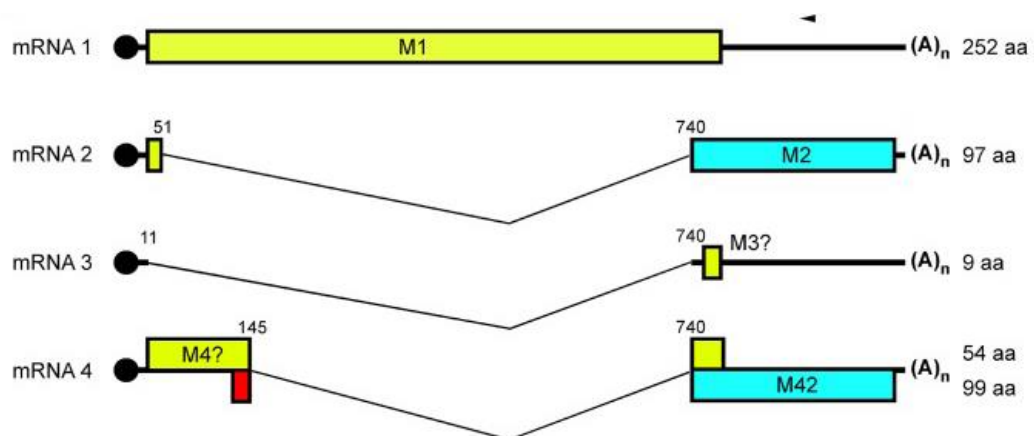


Figure 1.3 IAV segment 7 mRNA splice variants. 5'-splice donor (SD) and 3'-splice acceptor (SA) site positions are indicated and total sizes (a.a.) of the proteins they originate are specified on the right. The potential ORFs are colour coded with yellow corresponding to sequences in common with M1, blue to sequences specific to M2 and red to the sequence specific to M42. Adapted from (Wise et al., 2012).

role for the viral polymerase in regulating splicing mentioned above, it is clear that single nucleotide differences in the regions of the various splice donor sites can have large effects on their relative usage (Hutchinson et al., 2008; Shih et al., 1995; Wise et al., 2012). It is also possible that other sequences within segment 7 mRNA can influence the levels of splicing. For example, the identification of two possible RNA conformations in the region of the 3' splice site of segment 7, a hairpin or a pseudoknot, lead to the suggestion that a conformational switch that would expose or hide the splice site could play a role in segment 7

splicing regulation (Moss et al., 2012). The viral protein NS1 has been suggested to play a role in regulation of segment 7 splicing although not all studies are in agreement. For instance, NS1 has been proposed to inhibit splicing of segment 7 mRNA (Lu et al., 1994) while no effect on segment 7 splicing was observed in the absence of NS1 in another report (Salvatore et al., 2002). Conversely, one study has shown that the absence of NS1 did alter the accumulation of segment 7 spliced mRNAs, suggesting that NS1 played a regulatory role in segment 7 splicing (Robb and Fodor, 2012). More recently, further evidence has emerged in support of NS1 regulating segment 7 splicing by mediating the trafficking of segment 7 mRNA through host nuclear speckles (Mor et al., 2016). In this study, NS1 was proposed to interact with NS1-BP and direct unspliced segment 7 mRNA to nuclear speckles, which are storage sites for cellular splicing factors. Both segment 7 spliced mRNA2 and mRNA3 were found in nuclear speckles and this localisation was reduced in the absence of NS1 protein. According to this model, NS1 promotes post-transcriptional splicing of M1 mRNA during infection in a heterogeneous nuclear ribonucleoprotein K (hnRNP K)-dependent manner. Reduced levels of NS1-BP and hnRNP K were previously found to alter M2/M1 mRNA and protein ratios (Tsai et al., 2013).

Since IAV transcription occurs in the cell nucleus, mature viral mRNAs must be exported to the cytoplasm in order to be translated into proteins. IAV mRNAs have been suggested to require the cellular export pathway TAP/NXF1 in order to be exported from the nucleus (Hao et al., 2008; Read and Digard, 2010; Satterly et al., 2007; Wang et al., 2008). Since this thesis focuses on IAV mRNA export, both cellular and viral mRNA export mechanisms will be discussed in detail in individual sections, later in this chapter.

1.4.3 Genome replication

Once vRNPs are imported into the cell nucleus, replication of the genome can commence, in a two-step process. In the first step, a positive-sense intermediate “cRNA” molecule is synthesised and used in the second step as a template for the production of exact copies of the genomic vRNA (Neumann et al., 2004; Te Velhuis and Fodor, 2016).

The polymerase subunit PB1 initiates cRNA synthesis at the 3' end of vRNA (Gonzalez and Ortin, 1999; Li et al., 1998a) and catalyses the chain elongation reaction (Braam et al., 1983). Unlike mRNA, cRNA synthesis occurs in a primer-independent fashion (Hay et al., 1982) and does not involve premature termination or polyadenylation. Therefore, the resulting cRNA molecules are full-length copies of the vRNA containing non-coding regions at both ends that will act as promoters for vRNA synthesis (Palese and Shaw, 2007).

In the second step of replication, vRNA is synthesised using cRNA molecules as template. Unlike initiation from a vRNA template which is primer-independent, initiation from a cRNA template occurs via the synthesis of a dinucleotide (ApG) within the 3' promoter region. The dinucleotide is then realigned to the 3' end of the cRNA to act as a primer for full-length vRNA synthesis (Deng et al., 2006). Furthermore, replication from a cRNA template *in vitro* relies on the presence of a second RdRp that is not required to be catalytically active and thus thought to be responsible for the trans-activation of the resident RdRp (York et al., 2013).

Since both genome transcription and replication are carried out by the viral polymerase, a regulation of these processes is necessary to ensure that levels of mRNA, cRNA and vRNA are produced in sufficient amounts and at the appropriate times. It has been proposed that soluble NP could be a factor required for this switch (Beaton and Krug, 1986; Shapiro and Krug, 1988). However, another model suggests that the initiation of mRNA or cRNA synthesis could be a stochastic process that does not depend on a switch to regulate the transition between transcription and replication. Rather, NP and the RdRp would stabilise nascent cRNA molecules that would otherwise be degraded by host cell nucleases (Vreede et al., 2004). While several viral and cellular factors have been implicated or associated with viral RNA synthesis (Te Velthuis and Fodor, 2016), the mechanism by which the transition between transcription and replication occurs remains unclear.

1.4.4 Nuclear export of vRNPs

Following genome replication, progeny vRNPs are exported into the cytoplasm in a process that involves the participation of M1 and NS2 proteins (Cros and Palese, 2003). vRNPs are far too large to simply diffuse through nuclear pores, so instead the virus hijacks the cellular chromosome region maintenance 1 (CRM1) nuclear export pathway. Both M1 and NS2 are essential to this process, acting as a two-component adaptor between vRNPs and CRM1. M1 has long been known to bind to vRNPs (Zvonarjev and Ghendon, 1980) and in the absence of M1, vRNP export fails (Martin and Helenius, 1991). Although M1 is not required for vRNP formation (Huang et al., 1990), it has been proposed to promote the assembly of vRNPs into an export-competent conformation (Huang et al., 2001). The NS2 protein on the other hand, has been shown to interact with the CRM1 export receptor (Neumann et al., 2000), nucleoporins (O'Neill et al., 1998) and M1 protein (Akarsu et al., 2003; Yasuda et al., 1993). It has been thus proposed that a complex of vRNP, M1 and NS2 is formed and exported to the cytoplasm via the cellular export pathway CRM1 (Neumann et al.,

2000; O'Neill et al., 1998). The necessity of CRM1 for RNP export was established primarily by studies using a specific fungal inhibitor of it, Leptomycin B (LMB), which found that cells treated with this drug failed to export RNPs (Elton et al., 2001; Ma et al., 2001; Watanabe et al., 2001). Once exported from the nucleus, vRNPs are directed to the pericentriolar recycling endosome to get access to the cellular Rab-11 dependent vesicular transport pathway towards the apical plasma membrane (Amorim et al., 2011; Chutiwitoonchai and Aida, 2016; Kawaguchi et al., 2012; Momose et al., 2011).

1.4.5 Assembly and budding

Following replication of vRNA, transcription of viral mRNA and translation of viral proteins, the components that will form new virions must be assembled and packaged into individual viral particles. These processes are crucial for the release of progeny virions, through budding which occurs from the apical membrane of infected cells (Rodriguez Boulan and Sabatini, 1978).

The viral proteins that are found in the viral envelope: HA, NA and M2 are synthesised by membrane-bound ribosomes in the rough endoplasmic reticulum (Doms et al., 1993). HA and NA proteins are glycosylated in the Golgi network and use the *trans*-Golgi membrane to reach the apical plasma membrane where they accumulate in lipid raft microdomains (Barman et al., 2001; Scheiffele et al., 1999; Zhang et al., 2000). M2 is also a transmembrane protein found at the surface of infected cells (Lamb et al., 1985), but not in the lipid microdomains where HA and NA concentrate (Leser and Lamb, 2005; Zhang et al., 2000). Rather, M2 has been found in the periphery of lipid raft domains (Rossman et al., 2010; Thaa et al., 2011) still associated with the budding sites of infected cells (Rossman et al., 2010; Schroeder et al., 2005). The remaining virion components are thought to reach the site of viral assembly using the actin cytoskeleton and/or the microtubule network (Amorim et al., 2011; Avalos et al., 1997; Bucher et al., 1989; Digard et al., 1999). NS2 protein was initially thought to be a non-structural protein but was later found in virus particles (Richardson and Akkina, 1991; Yasuda et al., 1993); something that may reflect its association with RNPs and its role in their nuclear export.

The interior structure of the virion is thought to be formed via interactions between M1 and the cytoplasmic tails of HA and NA that then serves as a docking site for vRNP recruitment (Ali et al., 2000; Rossman and Lamb, 2011; Zhang et al., 2000). An interaction between M1 and the cytoplasmic tail of M2 has also been demonstrated and proposed to play an important role in viral assembly (Chen et al., 2008). M1 has furthermore been shown to

bind NP, in the form of vRNPs (Bui et al., 1996; Noton et al., 2007); thus being proposed to act as a bridge between vRNPs in the inner core of the virion and the membrane proteins at the surface (Nayak et al., 2004; Schmitt and Lamb, 2005). While vRNPs are believed to reach to apical plasma membrane using the cellular Rab-11 pathway (Amorim et al., 2011; Momose et al., 2011), one copy of each of the eight IAV genomic segments must be incorporated in the new virion. The selection of individual segments is thought to be mediated by *cis*-acting RNA sequences at the termini of vRNAs that act as packaging signals (Hutchinson et al., 2010). More recently, NS1 protein was identified in purified virions and suggested to also be part of the composition of the virus even though it has long been considered a non-structural protein (Hutchinson et al., 2014). However, how NS1 is recruited to the plasma membrane and what role it plays in viral assembly and/or budding remains unclear. In addition to viral components, a wide range of cellular factors have also been shown to be incorporated into virions (Hutchinson et al., 2014; Shaw et al., 2008), although this aspect of the viral assembly is still poorly understood.

Budding from the plasma membrane takes place once all the viral components have been assembled and results in the virions acquiring their final envelope when membrane scission occurs and the virions are released (Nayak et al., 2004; Schmitt and Lamb, 2005). Budding of IAV has been shown to require the Rab-11 pathway as virus particles failed to pinch-off from the plasma membrane in cells depleted of Rab11 (Bruce et al., 2010). Furthermore, the viral protein M2 has also been demonstrated to play a role in the final steps of the budding process, as mutations in this protein resulted in failure of membrane scission and virion release (Rossman et al., 2010). Finally, detachment of progeny virions from the host cell is ensured by the cleavage of sialic acid from cellular and viral membranes by NA, thereby preventing HA from binding the cell surface again through its interaction with sialic acid residues (Palese et al., 1974).

1.5 Cellular mRNA export

Protein synthesis primarily takes place in the cell cytoplasm and results from the translation of mRNAs which are produced in the nucleus. Hence once transcription is completed, mRNAs must be exported to the cytoplasm. However, prior to being fit for nuclear export, precursor mRNAs (pre-mRNAs) must turn into mature transcripts which involves undergoing multiple processing steps which are thought to occur co-transcriptionally (Bentley, 2002; Bentley, 2005). These processing steps include 5' end capping, removal of

introns through splicing mechanisms, cleavage and polyadenylation at the 3' end. All of them are carried out by the many different components of the mRNA processing machinery that join the RNA polymerase II transcription machine at 'transcription factories' (Bentley, 2002; Bentley, 2005; Howe, 2002). During mRNA processing, the transcript is always bound to a plethora of different proteins, forming messenger ribonucleoprotein particles (mRNPs). The proteins that are associated to the mRNA change throughout the maturation of the mRNA. For example, heterogeneous ribonucleoproteins associate with pre-mRNA, forming heterogeneous ribonucleoprotein particles (hnRNPs) during transcription and regulate splicing. Once splicing of the pre-mRNA has been carried out, the hnRNP proteins remain mainly associated to the spliced introns (Muller-McNicoll and Neugebauer, 2013).

Efficient mRNA nuclear export is coupled to processing steps through a series of protein-protein and protein-RNA interactions and the protein composition of a mature mRNA gives it the appropriate coating to undergo nuclear export, thereby ensuring that only mature mRNPs reach the cytoplasm for translation. The mechanism of cellular mRNA export has been extensively studied and defined in yeast and the pathway was found to be evolutionary conserved, being broadly applicable to higher eukaryotes (Okamura et al., 2015).

The transcription-export complex (TREX) has been suggested to have a conserved role in coupling transcription to mRNA export. In particular, TREX has been shown to be specifically recruited to nascent pre-mRNAs during transcription elongation (Strasser et al., 2002; Zenklusen et al., 2002). The TREX complex is formed by proteins Aly (THOC4), UAP56 (BAT1) and the human counterparts of a protein complex known to be involved in transcription in yeast, the THO complex (Strasser et al., 2002). Aly has been shown to interact with the nuclear RNA export factor 1 (NXF1), being recruited during splicing and resulting in an increased export efficiency for spliced mRNAs (Stutz et al., 2000; Zhou et al., 2000).

NXF1 has been identified as a bulk mRNA nuclear export factor as its overexpression in cell culture or microinjection into *Xenopus* oocytes stimulated efficient mRNA nuclear export (Braun et al., 2001; Herold et al., 2000; Yang et al., 2001). In order to promote the directional transport of mRNA across the nuclear pore complex (NPC), NXF1 forms a heterodimer with its co-factor Nxt1 (p15) to associate with nucleoporins at the NPC (Braun et al., 2002). It has been suggested that an intramolecular interaction inhibits NXF1's own RNA-binding activity and that its interaction with the Aly and Thoc5 components of the TREX complex results in a conformational change that exposes its RNA-binding domain and thus enables RNA-binding (Viphakone et al., 2012). Another TREX component that has also been

shown to be required for mRNA nuclear export is the helicase UAP56, which functions during spliceosome assembly (Fleckner et al., 1997; Zhang and Green, 2001). UAP56 has been demonstrated to interact specifically with Aly and both were found to be present together in spliced mRNPs, suggesting that one recruited the other (Luo et al., 2001). Inhibition of the interaction between UAP56 and Aly prevented the latter from being recruited to the mRNP (Luo et al., 2001). This is in line with other reports that couple mRNA nuclear export with a further mRNA processing step, splicing (Huang and Steitz, 2001; Luo and Reed, 1999; Zhou et al., 2000). Furthermore, UAP56 was shown to be required for the ATP-dependent interaction between Aly and THO and thus, UAP56 has been considered the core component of TREX (Dufu et al., 2010). In addition, both Aly and UAP56 have been associated with the Exon Junction Complex (EJC) which is deposited 20-24 nucleotides upstream of an exon-exon junction generated from intron removal (Le Hir et al., 2000). UAP56 has been shown to preferentially interact with spliced mRNAs carrying the EJC (Gatfield et al., 2001), while Aly is a component of the EJC which has been proposed to provide a binding platform for factors involved with mRNA export (Le Hir et al., 2001). Hence, the association of the TREX complex with the EJC was proposed to be the functional link between splicing and mRNA export (Strasser et al., 2002). UAP56 has also been linked to the NXF1/Nxt1 export pathway as a genome-wide analysis of nuclear mRNA export pathways in *Drosophila* revealed striking similarities in the mRNA expression profiles in NXF1, Nxt1 and UAP56 knock-out cells, indicating that these proteins operated in the same pathway (Herold et al., 2003). However, Aly has been found to be dispensable for mRNA nuclear export in metazoan (specifically, *Drosophila*) cells while NXF1 and UAP56 were found to be essential, indicating that other proteins other than Aly may act as adaptor proteins in this pathway (Gatfield and Izaurralde, 2002). In addition to transcription and splicing, the recruitment of TREX complex is also a cap-dependent process, as only 5' capped mRNAs were found associated with TREX (Cheng et al., 2006). The interaction found between Aly and the large subunit of the nuclear cap-binding complex (CBC), cap-binding protein 80 (CBP80) was suggested to recruit the TREX complex. The association between CBP80 and the TREX complex mediated by Aly would position TREX at the 5' end of the mRNA, resulting in its export through the nuclear pore in a 5' to 3' direction (Cheng et al., 2006).

Nuclear export of cellular mRNAs has also been linked to the 3' end processing of the mRNA that involves cleavage and polyadenylation of transcripts, although the precise mechanisms behind this association remain poorly understood. Abrogation of poly(A) synthesis at the mRNA 3' end has been shown to inhibit mRNA export in yeast and

mammalian cells (Dower et al., 2004; Huang and Carmichael, 1996; Libri et al., 2002). Furthermore, the deletion of *cis*-acting sequences in the 3' UTR of pre-mRNA required to couple 3' processing and termination in yeast resulted in transcripts failing to be exported from the nucleus (Hammell et al., 2002). In addition, several, but not all, export mutant mRNAs analysed in the same study were found to be improperly 3' processed transcripts (Hammell et al., 2002). Conversely, mutation of several mRNA export proteins in *Saccharomyces cerevisiae* resulted in hyperadenylation of transcripts, resulting in pre-mRNAs with longer poly(A) tails that rapidly accumulated in the nucleus at transcription sites, suggesting that mRNA maturation events determine mRNA export competence (Jensen et al., 2001). Moreover, the action of the THO and its associated helicase Sub2p (the yeast counterpart of human UAP56) has been suggested to occur after commitment to 3' end processing, which would coordinate events leading to efficient mRNA nuclear export (Rougemaille et al., 2008). In particular, this study found that THO/Subp2 mutations resulted in the specific sequestration of the 3' ends of a subset of genes within stalled RNP intermediates. Further evidence connecting mRNA export and 3' end processing emerged from a report suggesting that Yra1 (the yeast counterpart of Aly) was recruited by a direct interaction with the 3' end processing factor Pcf11, thereby coordinating export with 3' end processing (Johnson et al., 2009). Finally, NXF1 has been shown to interact with cleavage and polyadenylation specificity factor 6 (CPSF6) that is involved in mRNA polyadenylation (Ruepp et al., 2011). This export model is summarised in **Figure 1.4** and applies to the bulk of cellular mRNAs, which are, in their vast majority, spliced.

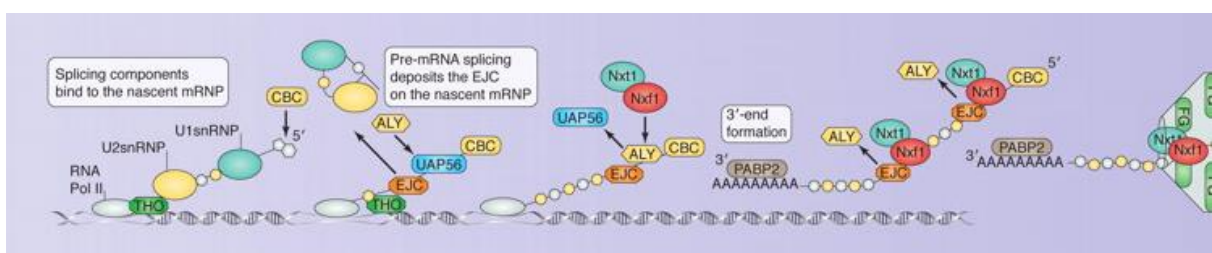


Figure 1.4 Summary of the current model for bulk mRNA nuclear export, which is achieved by the NXF1/Nxt1 export pathway in a sequential series of steps. Per this model, UAP56 recruits Aly which subsequently recruits and, acting as an adaptor protein, hands over the mRNA to NXF1, marking the mature transcript for nuclear export through the nuclear pore complex into the cell cytoplasm. Adapted from (Carmody and Wentz, 2009).

However, there are also cellular mRNAs which do not contain introns and mRNAs which have been shown to not use the NXF1/Nxt1 export pathway. The export of cellular mRNAs can be selective and dependent on specific *cis*-acting RNA elements and interactions with specific adaptor proteins (Delaleau and Borden, 2015). For instance, CRM1 is a member

of the importin β superfamily of nuclear transport receptors and has been established as required for the nuclear export of many RNAs (Fukuda et al., 1997; Hutten and Kehlenbach, 2007; Stade et al., 1997; Watanabe et al., 1999). While NXF1 requires an adaptor protein to increase its affinity with RNA (Viphakone et al., 2012), CRM1 is thought to interact directly with its mRNA cargoes via *cis*-acting RNA export elements (Fukuda et al., 1997; Popa et al., 2002). It has been suggested the RNA length could dictate which export pathway is used. In particular, it was found that sequences greater than 300 nucleotides, like mRNAs, almost exclusively use the NXF1 pathway, while sequences shorter than 120 nucleotides, such as U spliceosomal small nuclear RNAs (snRNAs), used a CRM1 pathway (Masuyama et al., 2004).

Other export strategies have been found for intronless transcripts and viral mRNAs are often used as tools to study the export mechanisms of this type of transcript. The metazoan replication-dependent histone mRNAs are not spliced or polyadenylated, but rather present a highly conserved stem-loop structure at the 3' end (Marzluff, 1992, 2005; Marzluff et al., 2008). The cytoplasmic accumulation of such mRNAs has been shown to be facilitated by a short *cis*-acting element in the protein coding region of the mRNA that facilitates their cytoplasmic accumulation, with a function analogous to the human immunodeficiency virus (HIV) protein Rev (Huang and Carmichael, 1997). Furthermore, when inserted into the 5' untranslated region of a human β -globin cDNA construct, this *cis*-acting sequence was shown to act as a polyadenylation enhancer and a potent inhibitor of splicing in addition to promoting mRNA export (Huang et al., 1999). The cellular stem-loop binding protein (SLBP) has been identified as a factor necessary for the nuclear export of histone mRNAs. SLBP has been demonstrated to interact with the histone mRNA and SLBP knockdown human bone osteosarcoma epithelial (U2OS) cells resulted in retention of the mRNA in the cell nucleus (Pandey et al., 1994; Sullivan et al., 2009). The NXF1 pathway has also been shown to be used for the export of intronless transcripts. It has been suggested that an unstructured RNA sequence is capable of recruiting EJC elements and thus the NXF1 export pathway, as adding 300-350 nucleotides of random mRNA exon sequences into U1 snRNAs resulted in the hybrid RNAs being exported like mRNAs (via NXF1) rather than like U1 snRNAs which are exported via the CRM1 pathway (Ohno et al., 2002). Furthermore, it has been proposed that intronless mRNAs, other than histone mRNAs, contain specific sequences that result in their efficient integration into the TREX mRNA complex and that the cytoplasmic accumulation of naturally intronless transcripts requires a polyadenylation signal, TREX components and NXF1 (Lei et al., 2011). Thus, even though the NXF1 pathway is mostly associated with the export of spliced mRNAs, splicing is not an absolute requirement for this pathway to be used.

1.6 IAV mRNA export

IAV genome replication and transcription occur in the cell nucleus which creates the necessity for both vRNA and mRNA to be exported to the cytoplasm. The nuclear export of vRNPs is well studied and, as described above, is achieved using the CRM1 cellular export pathway with the participation of viral proteins M1 and NS2 (Elton et al., 2001; Neumann et al., 2000; O'Neill et al., 1998; Paterson and Fodor, 2012). However, how IAV mRNAs reach the cell cytoplasm remains less understood. Viral mRNAs have a similar structure to cellular mRNAs as they possess a 5' cap structure and a 3' poly(A)_n tail although these features are acquired through different mechanisms (York and Fodor, 2013). As detailed in the previous section, cellular mRNA maturation that includes 5' capping, splicing and 3' end processing of active transcripts is coupled to their nuclear export. It is then possible that viral mRNAs mimic the structure of cellular mRNAs to gain access to the cellular export machinery, as well as to ensure their successful translation by ribosomes. In support of this view, IAV mRNA export has been shown to require the ongoing activity of RNAP II, as its inhibition resulted in mRNA nuclear retention (Amorim et al., 2007; Vogel et al., 1994). RNAP II was further demonstrated to interact with the IAV polymerase resulting in the ubiquitylation of RNAP II leading to its subsequent degradation in infected cells (Vreede and Fodor 2010). The association between the two polymerases would not only place transcribing viral RNPs in the correct sites in the nucleus to acquire 5' cap structures by snatching them from cellular mRNA, but also to be in close proximity with export cellular factors, namely NXF1. It is established that IAV mRNAs do not use the CRM1 pathway for their nuclear export (Amorim et al., 2007; Elton et al., 2001; Larsen et al., 2014; Read and Digard, 2010). Instead, the view is that the virus uses the NXF1/Nxt1 export pathway, as several reports provide mounting evidence for its involvement. Initial evidence came from a *Drosophila* cell-based screen for host genes involved in IAV replication which indicated the involvement of NXF1 in IAV replication (Hao et al., 2008). NXF1 has been shown to interact with several, but not all, IAV mRNAs (Larsen et al., 2014; Satterly et al., 2007; Wang et al., 2008). Furthermore, siRNA-mediated depletion of NXF1 has been demonstrated to result in the inhibition of nuclear export of selected IAV transcripts and/or overall virus replication (Hao et al., 2008; Larsen et al., 2014; Read and Digard, 2010). Reduced levels of UAP56, which participates in the NXF1/Nxt1 export pathway, was also shown to result in defective nuclear export of some IAV transcripts (Mor et al., 2016; Read and Digard, 2010). The requirement of Aly for IAV mRNA export is less consensual, as one study found that that depletion of Aly had little effect on mRNA export (Read and Digard, 2010) while another suggested that the knockdown of Aly inhibited export of M1 and M2 mRNAs (Mor et al., 2016). A further study found that

IAV segment 6 mRNA interacted with both the CBC and Aly, again suggesting that viral mRNAs followed the same pathway for nuclear export as cellular mRNAs (Bier et al., 2011).

While the involvement of NXF1 in IAV mRNA nuclear export is established, there is also evidence that this cellular factor is not absolutely required for the export of all IAV mRNAs and that mRNAs from different segments use different strategies to reach the cytoplasm. For instance, the various IAV mRNAs have shown distinct degrees of sensitivity to the depletion of NXF1. While the nuclear export of intronless late gene transcripts from segments 4 and 6 as well as unspliced segment 7 showed a high degree of dependence on NXF1, export of intronless early gene mRNAs from segments 1, 2, 3 and 5 showed little dependency on this factor (Larsen et al., 2014; Read and Digard, 2010). The use of selective mRNA export mechanisms by different segments is also in line with a previous study that showed that the ongoing activity of RNAP II was required for the nuclear export of segment 7 but not segment 5 mRNA (Amorim et al., 2007). Presumably the selection of mRNA export strategy is related to interactions between viral mRNAs and particular cellular factors, likely dictated by *cis*-acting sequences specific to each transcript, but this remains to be elucidated. More recently, it has been proposed that the RNA helicase DDX19 (normally resident on the cytoplasmic face of the NPC) is recruited by the viral polymerase to promote the nuclear export of all classes of viral transcripts, intronless, spliced and unspliced (Diot et al., 2016). This study suggested that the viral polymerase recruits DDX19 in an RNA-independent manner and that the latter associated with viral transcripts to enhance their export, as DDX19 depletion resulted in highly reduced levels of cytoplasmic viral mRNA. However, depletion of DDX19 also reduced overall levels of viral transcription and the exact involvement of DDX19 in the nuclear export of IAV mRNAs is unclear.

The viral protein NS1 has also been hypothesised to participate in IAV mRNA nuclear export (Schneider and Wolff, 2009) and its involvement in this process is the main focus of this dissertation. NS1 has been shown to interact and co-localise with NXF1 and IAV transcripts, suggesting that it could play a role in IAV mRNA nuclear export (Wang et al., 2008). Recently (after the work reported here was completed), it has been proposed that NS1 mediates M1 mRNA transport through nuclear speckles, promoting the splicing of segment 7 and the nuclear export of M2 mRNA (Mor et al., 2016). However, it remains unclear how NS1 could play a role in the nuclear export of intronless or unspliced mRNAs. Even though NS1 has been suggested to play a role in IAV mRNA nuclear export before (Schneider and Wolff, 2009) and some subsequent reports support this argument, there are also several

studies suggesting that NS1 has the opposite effect on nuclear export of cellular mRNAs (Fortes et al., 1994; Garaigorta and Ortin, 2007; Qian et al., 1994; Qiu and Krug, 1994). Specifically, NS1 was shown to inhibit the nuclear export of mRNAs containing a poly(A) tail (Qiu and Krug, 1994), potentially by interacting with CPSF30 and thereby inhibiting the 3' end formation of cellular mRNAs (Nemeroff et al., 1998). Another report suggested that NS1 interacted with poly(A) binding protein II (PABPII) which resulted in inhibition of the poly(A) tail elongation (Chen et al., 1999). In general, these results can be summarised as reflecting an export block resulting from inhibition of pre-mRNA maturation, rather than a direct effect on the mRNA export apparatus itself. However, more recently, NS1 has been proposed to directly target the cellular mRNA export machinery and the nuclear pore complex with the function of inhibiting transport of cellular mRNAs (Satterly et al., 2007). In this study, results indicated that NS1 could interact with NXF1, Nxt1 and other components of the RNA nuclear export machinery such as Rae1 and E1B-AP5. The analysis of NS1 deletion mutants revealed that Nxt1 interacted with the carboxyl-terminal domain of NS1 while NS1 interaction with NXF1, Rae and E1B-AP5 required both the amino- and carboxyl-terminal domains of NS1. All these interactions aside from the one between NS1 and E1B-AP5 were shown to occur in an RNA-independent manner. In addition, an interaction between nucleoporin98 (Nup98) and NS1 was also demonstrated (Satterly et al., 2007).

Nevertheless, the thought that NS1 could act as an adaptor protein with the role of promoting viral mRNA through a cellular export pathway is not unfounded and has precedents, as other viruses have been shown to use similar strategies to export their mRNAs. The classic example of this is the human immunodeficiency virus- 1 (HIV-1) utilising its Rev protein as an adaptor to direct its singly-spliced and unspliced mRNAs into the CRM1 export pathway (Cullen, 2003). Potentially more relevant to IAV and its reliance on the NXF1/Nxt1 pathway, herpes simplex virus type 1 (HSV-1) protein ICP27 was shown to promote the nuclear export of intronless viral mRNAs by interacting with Aly in order to access the NXF1/Nxt1 export pathway (Chen et al., 2002). However, Aly was subsequently found to be dispensable for the export of HSV-1 intronless mRNAs (Johnson et al., 2009), indicating that redundant mechanisms may be used by viruses to export their mRNAs. An example of such a redundant mechanism was found in Kaposi's sarcoma-associated herpesvirus (KSHV) where, in the absence of Aly, ORF57 (the homologue of HSV-1 ICP27) was able to interact with another cellular protein, UIF, and thereby recruit the TREX complex and NXF1 onto KSHV intronless mRNAs (Jackson et al., 2011).

1.7 IAV NS1 protein

NS1 protein is the gene product resulting from segment 8 unspliced mRNA translation (**Figure 1.5**). It is commonly referred to as a multifunctional protein due to the plethora of roles and functions attributed to it, often in a virus strain-dependent manner (Hale et al., 2008b). Its length varies according to the virus strain, generally ranging from 218-237 a.a. (although shorter forms are found) and it shares ten N-terminal a.a. with NS2, the gene product of segment 8 spliced mRNA (Abdelwhab el et al., 2016; Lamb and Lai, 1980; Palese and Shaw, 2007). It was accepted for many years that NS1 is a non-structural component of IAV virions, hence its name. However, this has been recently disputed as NS1 was identified in preparations of purified virions, suggesting that it is present in the core structure of the virus (Hutchinson et al., 2014). In infected cells, the amount of NS2 mRNA was found to be 10% compared to levels of NS1 mRNA (Lamb et al., 1980). As discussed above, NS1 has been suggested to regulate splicing from the very segment it originates from in order to control the amounts of NS1 and NS2 proteins produced during infection (Chua et al., 2013; Garaigorta and Ortin, 2007). Two distinct functional domains have been identified in NS1, an N-terminal RNA binding domain (residues 1-73) and a C-terminal effector domain (residues 74-230) (Hale et al., 2008b). These domains were originally defined biochemically and functionally, but have since been resolved at the atomic level in a variety of high resolution structures that collectively show an alpha-helical RNA binding domain separated from an effector domain, largely comprised of beta-sheet, by a short linker region (Bornholdt and Prasad, 2006; Dundon and Capua, 2009; Kerry et al., 2011b; Liu et al., 1997).

The RNA-binding domain has been shown to interact with several RNA species such as double-stranded RNAs, snRNAs, viral genomic RNAs and viral mRNAs (Chien et al., 1997; Hatada and Fukuda, 1992; Hatada et al., 1997; Hatada et al., 1992; Marion et al., 1997; Qiu et al., 1995; Wang and Krug, 1998; Wang et al., 1999). Two residues were identified as essential for this domain to remain active: an arginine at position 38 and a lysine at position 41, as substitution of these residues was shown to abrogate the NS1 RNA-binding activity (Wang et al., 1999). The effector domain is thought to be mainly responsible for mediating interactions with host factors and it has also been shown to stabilise the RNA-binding domain, while both domains contribute to the multimerisation of full-length NS1 (Hale et al., 2008a; Nemeroff et al., 1995; Wang et al., 2002). During infection, NS1 can be found both in the cell nucleus and in the cytoplasm (Greenspan et al., 1988; Newby et al., 2007). The presence of both nuclear localisation sequences (NLS) (Greenspan et al., 1988; Melen et al.,

2007) as well as a nuclear export signal (NES) (Li et al., 1998b) in NS1 suggests that it may have the ability to shuttle between the nucleus and the cytoplasm (Li et al., 2015).

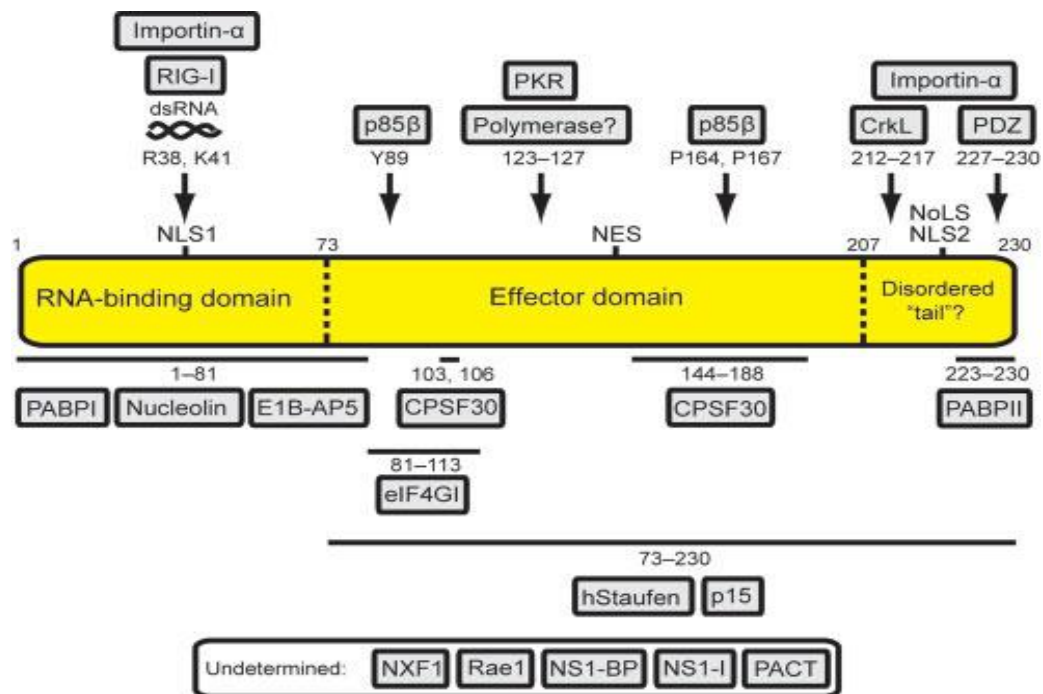


Figure 1.5 Representation of IAV NS1 protein. The dashed lines indicate the boundaries of the functional domains of the protein and their positions (a.a.) specified on the top, along with the signals of the NLSs, a nucleolar localisation signal (NoLS) and NES. Known interactions between NS1 and cellular factors are depicted at the relative position of the residues thought to be critical for each particular interaction. The N-terminus of NS1 includes the RNA-binding domain which interacts with multiple RNA species. The C-terminus encompasses the effector domain which is responsible for the majority of the known interactions between NS1 and cellular factors. This domain is followed by around 20 a.a. which are possibly unstructured and referred to as a “disordered tail”, also associated with interactions with components of the cellular machinery. Figure taken from (Hale et al., 2008b).

The major function credited to NS1 is the one of antagonizing host immune responses (Egorov et al., 1998; Garcia-Sastre et al., 1998; Kochs et al., 2007). It was found that influenza A viruses that did not express NS1 induced large amounts of interferon (IFN) and replicated more efficiently in IFN- α/β deficient systems such as Vero cells, even though their virus titres were still lower when compared to WT viruses expressing NS1 (Garcia-Sastre et al., 1998; Kochs et al., 2007). Although antagonism of innate immune functions is regarded as the primary role of NS1, it has been shown to play many other roles throughout the IAV life cycle, through numerous protein-RNA and protein-protein interactions. For instance, NS1 interferes with many host processes at different stages of viral infection (Khaperskyy and McCormick, 2015). As already discussed, early in infection, NS1 has been shown to interfere with the 3' end processing of cellular mRNAs, resulting in the inhibition of mRNA

polyadenylation in the cell nucleus (Nemeroff et al., 1998). Later in infection, NS1 can also be found in the cytoplasm where it inhibits the antiviral proteins 2'-5' oligo(A) synthetase (OAS) and protein kinase R (PKR) by interfering with their ability to bind dsRNA (Garcia et al., 2006; Silverman, 2007). NS1 has been demonstrated to out-compete OAS for interaction with dsRNA (Min and Krug, 2006) and proposed to interact with PKR and thereby inhibit the conformational change thought to be necessary for PKR auto-inhibition (Li et al., 2006). Residues at positions 35 and 36 in NS1 have recently been identified as critical for blocking the activation of PKR, as their mutation abrogates the ability of NS1 to interact with PKR and dsRNA (Schierhorn et al., 2017). Both these NS1 interactions protect viral mRNA from being targeted for degradation, ensuring their undisturbed translation in the cell cytoplasm.

NS1 protein has also been linked to actively enhancing translation of viral mRNAs. In support of this view, a study showed that the presence of NS1 resulted in higher accumulation levels of viral proteins NP and M1 in the cell cytoplasm and this NS1-mediated effect was attributed to an increase in the translation initiation rate (de la Luna et al., 1995). Subsequently, IAV gene expression was shown to be stimulated by the presence of NS1 in a study where a short sequence upstream of the initiation codon on segment 7 was found to be required for this NS1-associated effect (Enami et al., 1994). In this instance, it was suggested that NS1 acted at the level of translation since the amounts of M1 mRNA were not altered by NS1 expression. However, a direct effect on translation specifically as opposed to post-transcriptional processes was not demonstrated. NS1 was further shown to interact with the eukaryotic initiation factor 4G1 (eIF4G1) which is the large subunit of the cytoplasmic CBC, eIF4F, in an RNA-independent manner (Aragon et al., 2000). This study proposed that NS1 recruits eIF4G1 to the 5'UTR of IAV mRNAs, thereby favouring their translation. On the other hand, it has also been reported that viral mRNA translation was not significantly impaired in cells infected with an influenza virus that did not express NS1 in IFN-deficient systems (Salvatore et al., 2002). The findings in this study suggest that, while NS1 may enhance viral mRNA translation, this role is not essential for the replication of the virus.

Another role attributed to NS1 is the ability to activate the PI3K signalling pathway. NS1 was found to interact with the p85 β regulatory isoform of PI3K and several residues in NS1 have been implicated in this interaction (Hale et al., 2006; Li et al., 2008; Shin et al., 2007). Inhibition of PI3K was found to result in impaired propagation of IAV and greater induction of apoptosis in infected cells (Ehrhardt et al., 2006; Ehrhardt et al., 2007; Hale et al., 2006; Zhirnov and Klenk, 2007). NS1 could then play a role in limiting host-cell

apoptosis and thus favouring viral proliferation, although the implication of PI3K activation in the context of IAV infection remains poorly understood and warrants further investigation.

Even though the NS1 functions described above are considered the main ones, several other roles and multiple interactions with cellular factors have been associated to this viral protein (Hale et al., 2008b; Marc, 2014). In this dissertation, the role of NS1 in viral mRNA export was explored using several mutant NS1 proteins predicted to have loss of function (or in one case, gain of function) phenotypes affecting their ability to interfere with the various cellular functions above. Further details on these mutant polypeptides are given later in Chapter 3.

1.8 Aims of this study

This study is the continuity and further expansion of previous work performed in our laboratory (Amorim et al., 2007; Read and Digard, 2010). It focuses on understanding how IAV transcripts are exported from the host cell nucleus to the cytoplasm, starting from the hypothesis that NS1 could act as an adaptor between viral mRNAs and the cellular NXF1/Nxt1 mRNA export pathway. The overall goal of this dissertation was to determine the viral requirements for mRNA nuclear export and try to determine which cellular components are involved in the viral mRNA export mechanism. One chapter of this dissertation is aimed at identifying the viral requirements for segment 7 mRNA export, by analysing the cellular localisation of the mRNA by fluorescence in situ hybridisation (FISH) in the absence or presence of different viral proteins. The next two chapters of this dissertation are aimed at understanding the mechanism by which nuclear export of segment 7 occurs by using mutant viruses and testing interactions between viral proteins, viral mRNA and cellular proteins. The final chapter of this dissertation focuses on the goal of determining whether the viral and cellular requirements for segment 7 nuclear export are shared with other segments.

CHAPTER 2 – INVESTIGATING THE VIRAL REQUIREMENTS FOR mRNA NUCLEAR EXPORT

2.1 Introduction

IAV mRNA nuclear export is currently poorly understood and, in comparison to other aspects, not much is known about the requirements or the mechanisms for this vital process in the virus life cycle.

It has been established that IAV utilizes cellular machinery to export its transcripts. For instance, nuclear export of viral late gene mRNAs was shown to require the ongoing activity of DNA-dependent-RNA-polymerase II (RNAP II) at a post-transcriptional level. Inhibition of processive transcription by RNAP II using the drug DRB rather than the complete activity of the protein suggested that RNAP II was necessary not just to provide a source of 5'-cap structures for the viral polymerase but also played a role in facilitating viral mRNA export (Amorim et al., 2007). Consistent with this, the NXF1 pathway has been proposed to be involved in influenza A viral mRNA nuclear export (Hao et al., 2008; Read and Digard, 2010; Satterly et al., 2007; Wang et al., 2008). However, different segments showed different levels of dependence on NXF1 to achieve export (Larsen et al., 2014; Read and Digard, 2010) and how NXF1 is recruited to viral mRNAs remained unknown.

Previous work from the Digard lab suggested that the intracellular localisation of segment 7 mRNA analysed by fluorescent *in situ* hybridization (FISH) in the context of an RNP reconstitution assay was different from the one observed in infected cells. In infected cells, the mRNA was found in the cytoplasm as would be expected (Amorim et al., 2007; Read and Digard, 2010). However, it was seen to be retained in the cell nucleus when expressed in a minireplicon system utilising segment 7 as a vRNA template (Read, 2010), suggesting that a viral factor found in infected cells, but not in the minireplicon system, might be necessary for efficient export.

The aim of the research in this chapter was therefore to investigate potential role(s) for viral polypeptides in directing segment 7 viral mRNA export.

2.2 Results

2.2.1 Segment 7 mRNA localisation in infected vs. transfected cells

As outlined above, preliminary evidence (from a previous PhD student in the group) suggested that segment 7 mRNA localisation might differ between authentic virus infection and a minireplicon setting that expresses only a subset of the viral polypeptides. To test if this finding was reproducible, 293T cells were either infected with wild type A/PR/8/34 (WT PR8) virus or transfected with the basic minireplicon components (PB2, PB1, PA and NP) and a plasmid expressing segment 7 as the vRNA template. Cells were fixed and processed for FISH using a fluorescently labelled RNA probe to detect segment 7 positive sense RNA molecules. IAV produces two types of positive sense RNA – mRNA and cRNA – and the probes used are thus capable of hybridizing with both. However, since 90-95% of positive sense IAV RNA is estimated to correspond to mRNA transcripts (Elton et al., 2006), these are expected to be the majority of molecules detected by the probes. In addition, segment 7 is a spliced segment and several mRNAs can be synthesised from it: an unspliced transcript encoding for M1 protein and three spliced ones (mRNAs 2-4) (Inglis and Brown, 1981; Lamb and Choppin, 1981; Lamb et al., 1981; Shih et al., 1998). The spliced mRNA2 and mRNA4 have been shown to encode for the M2 and M42 proteins respectively while no gene product is known for mRNA3 (Wise et al., 2012). A probe specific to positive sense RNA from segment 7 will hybridise with all mRNAs generated from this segment but the majority of transcripts produced correspond to the unspliced mRNA (Lamb et al., 1981; Shih et al., 1998; Wise et al., 2012). Cells were also stained with DAPI to define the nucleus. In mock infected cells, no red staining was observed confirming that the FISH signal was specific to viral and not cellular mRNA (**Figure 2.1A**). PR8 infected cells showed abundant staining mainly of the cytoplasm, confirming successful infection and the expected (Amorim et al., 2007; Read and Digard, 2010) localisation of the segment 7 mRNA. When cells were transfected with four of the five minireplicon components (missing PB2 as a ‘2PNP’ negative control in which no mRNA synthesis is possible), only background levels of staining were observed (**Figure 2.1B**). In contrast, with the complete minireplicon set (3PNP), FISH signal was readily detected. In this sample however, segment 7 mRNA was largely retained in the cell nucleus. These results were in agreement with the initial observation made in the Digard laboratory and constituted the base for this dissertation. The fact that segment 7 mRNA was cytoplasmic in infected cells but retained in the cell nucleus upon transfection strongly suggested that there

was a viral factor missing from the minireplicon set that was required for efficient segment 7 mRNA nuclear export.

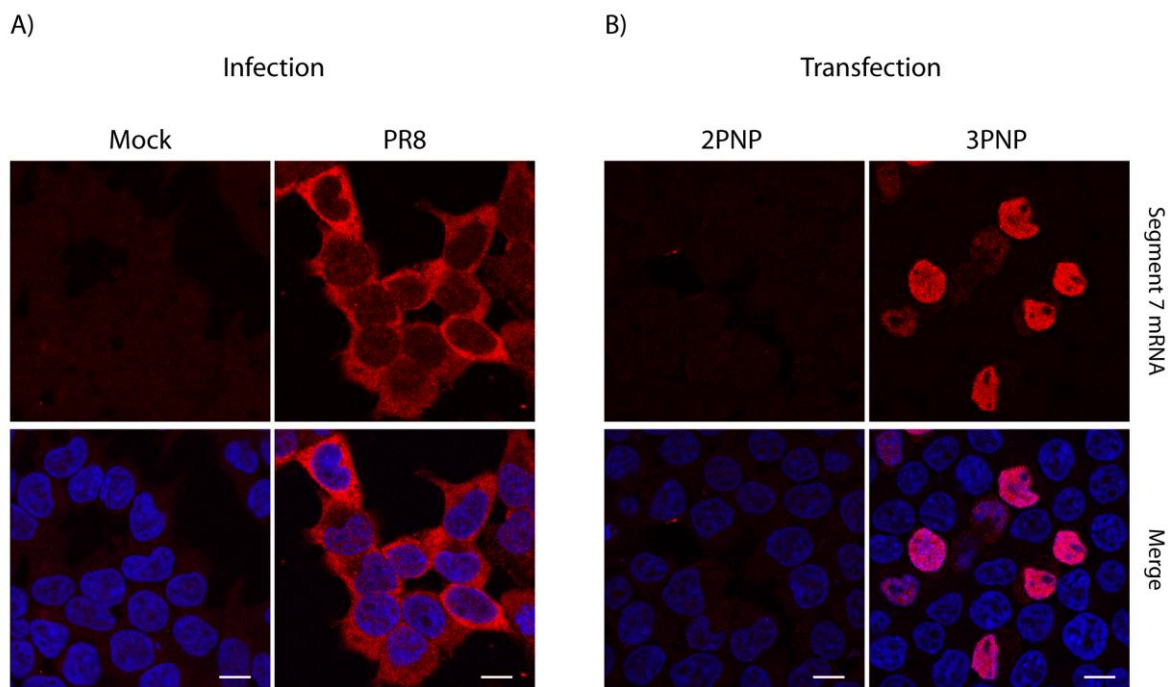


Figure 2.1 Intracellular localisation of segment 7 mRNA in virus infected cells versus cells transfected with plasmids for RNP reconstitution. A) 293T cells were either mock or infected with WT PR8 at an MOI of 5. B) 293T cells were co-transfected with plasmids expressing each of the IAV polymerase proteins and NP protein (3PNP), or plasmids expressing PB1, PA and NP protein (2PNP), along with a plasmid expressing an artificial segment 7 vRNA. At 6 h.p.i. (A) and 24 h.p.t (B) cells were processed for FISH analysis using Cy3 labelled RNA probes for positive sense viral RNAs from segment 7 (red) and counterstained with DAPI (blue) to indicate the position of the nucleus. Images were captured using a Leica-TCS confocal microscope and Leica TCS analysis software. Scale-bar indicates 10 µm.

2.2.2 Segment 7 mRNA localisation in the presence of further viral components

Starting from the premise that a viral factor that was not included in the basic minireplicon set was required for efficient segment 7 mRNA nuclear export, further viral components were added to the basic segment 7 minireplicon system. Missing components were products from segments 4, 6 and 8. Each of these segments was added (as plasmids with the appropriate cDNA under the control of a Pol I promoter) to the transfection mix individually and the effect of each addition on segment 7 mRNA localisation analysed by FISH. As before, a 2PNP negative control lacking PB2 was included and no red staining corresponding to segment 7 mRNA was observed in this sample (**Figure 2.2A**). A sample with the basic segment 7 set without further additions was used as a positive control and here, the FISH signal was mostly visible in the nucleus as shown previously (**Figure 2.1.B**). Subsequent addition of either segment 4 or 6 had no effect on segment 7 mRNA localisation

(**Figure 2.2A**) and thus the gene products from these segments were incapable of restoring the cytoplasmic mRNA phenotype seen in PR8 infected cells. Addition of segment 8 however resulted in a marked shift of segment 7 mRNA from the nucleus to the cell cytoplasm. To

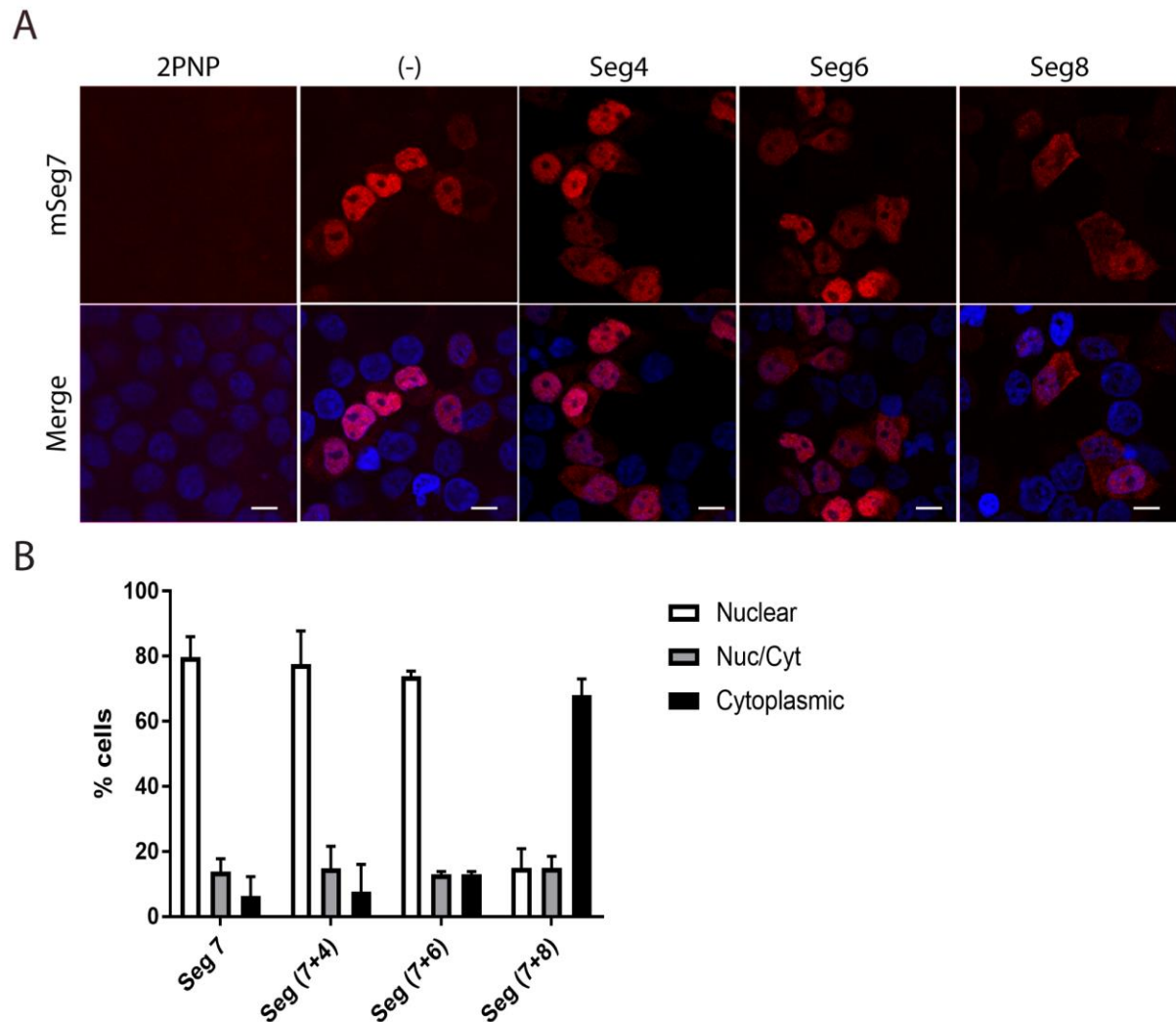


Figure 2.2 Testing the effect of individual segment additions to the segment 7 RNP reconstitution assay on the localisation of segment 7 mRNA. A) 293T cells were co-transfected with plasmids expressing each of the influenza polymerase proteins and NP protein (3PNP) or plasmids expressing PB1, PA and NP proteins (2PNP) alongside plasmids expressing vRNAs of the indicated segments. Cells were incubated and 24 h subsequently processed for FISH analysis using Cy3 labelled RNA probes for segment 7 positive sense RNAs (red). Images were captured using a Leica-TCS confocal microscope and Leica TCS analysis software. Scale-bar indicates 10 μ m. B) Cells expressing segment 7 mRNA were counted and scored according to the cellular localization of this mRNA: predominantly nuclear, a mixture of nuclear and cytoplasmic (nuc/cyt) or predominantly cytoplasmic. A minimum of 100 cells were scored for each sample. Data are the mean \pm SEM from two to six independent experiments.

provide a numerical estimate of the reproducibility of these effects, multiple cells from replicate experiments were scored as displaying predominantly nuclear, nuclear/cytoplasmic or predominantly cytoplasmic localisation of segment 7 RNA (**Figure 2.2B**). 80% of cells transfected with 3PNP and segment 7 on its own showed mRNA nuclear retention, ~15% had the mRNA localised to both the nucleus and the cytoplasm and the mRNA was found only in

the cytoplasm in very few (~5%) cells. After adding segment 4 to the set ~75% of transfected cells contained segment 7 mRNA in the nucleus, less than 20% had the mRNA in both cellular compartments and just over 5% of cells showed a cytoplasmic localization. Similar results were also obtained with segment 6. However, adding segment 8 to the RNP reconstitution assay caused a shift in localisation such that only ~15% of transfected cells showed segment 7 mRNA nuclear retention, ~15% of cells had the mRNA in both compartments and ~70 % had segment 7 mRNA localised mainly to the cytoplasm. Thus, the addition of segment 8 alone to the basic segment 7 RNP was sufficient to restore efficient nuclear export of segment 7 mRNA. The viral factor required for segment 7 mRNA nuclear export therefore lay within segment 8.

2.2.3 NS1 protein is required for efficient segment 7 mRNA nuclear export

IAV segment 8 encodes two proteins, NS1 and NS2, from unspliced and spliced mRNAs, respectively (Inglis et al., 1979; Lamb and Lai, 1980). Both of these would be expressed when segment 8 was added to the basic minireplicon set. Thus it was unknown which protein, specifically, was responsible for the shift in the cellular localisation of segment 7 mRNA observed in **Figure 2**. To distinguish between the effects of NS1 and NS2 expression on segment 7 mRNA localization, expression vectors for each of the proteins were separately added to the minireplicon set. The mRNA localisation was analysed by FISH as before. No staining was detected in the negative control 2PNP lacking the polymerase subunit PB2 and as before, segment 7 mRNA (stained in red) was seen to be retained in the nucleus when transcribed from only the 3PNP set of plasmids (**Figure 2.3A**). When IAV NS2 (NS2A) was added, segment 7 mRNA remained nuclear, indicating that NS2A protein was not sufficient to drive mRNA nuclear export. Upon addition of IAV NS1 (NS1A) however, a clear shift of segment 7 mRNA from the nucleus to the cytoplasm was observed, suggesting that NS1A protein was the IAV factor necessary to promote segment 7 mRNA nuclear export.

In order to investigate whether the ability of the IAV NS1 protein to promote segment 7 mRNA export was virus species-specific, an influenza B virus NS1 (NS1B) expression vector was tested. However, segment 7 mRNA staining was still detected in the cell nucleus after expression of NS1B (**Figure 2.3A**), indicating that it was not capable of promoting IAV mRNA export.

As mentioned above, segment 7 produces different forms of mRNAs due to splicing. While it is technically difficult to synthesize RNA probes specific to spliced mRNAs, it was possible to synthesise a probe that only contained the intron sequence of segment 7. This

allowed the study of M1 mRNA localisation in particular and thus to test whether there were differences between using a probe that was theoretically capable of hybridizing with all segment mRNA species and a probe that would only bind to the unspliced, M1 mRNA.

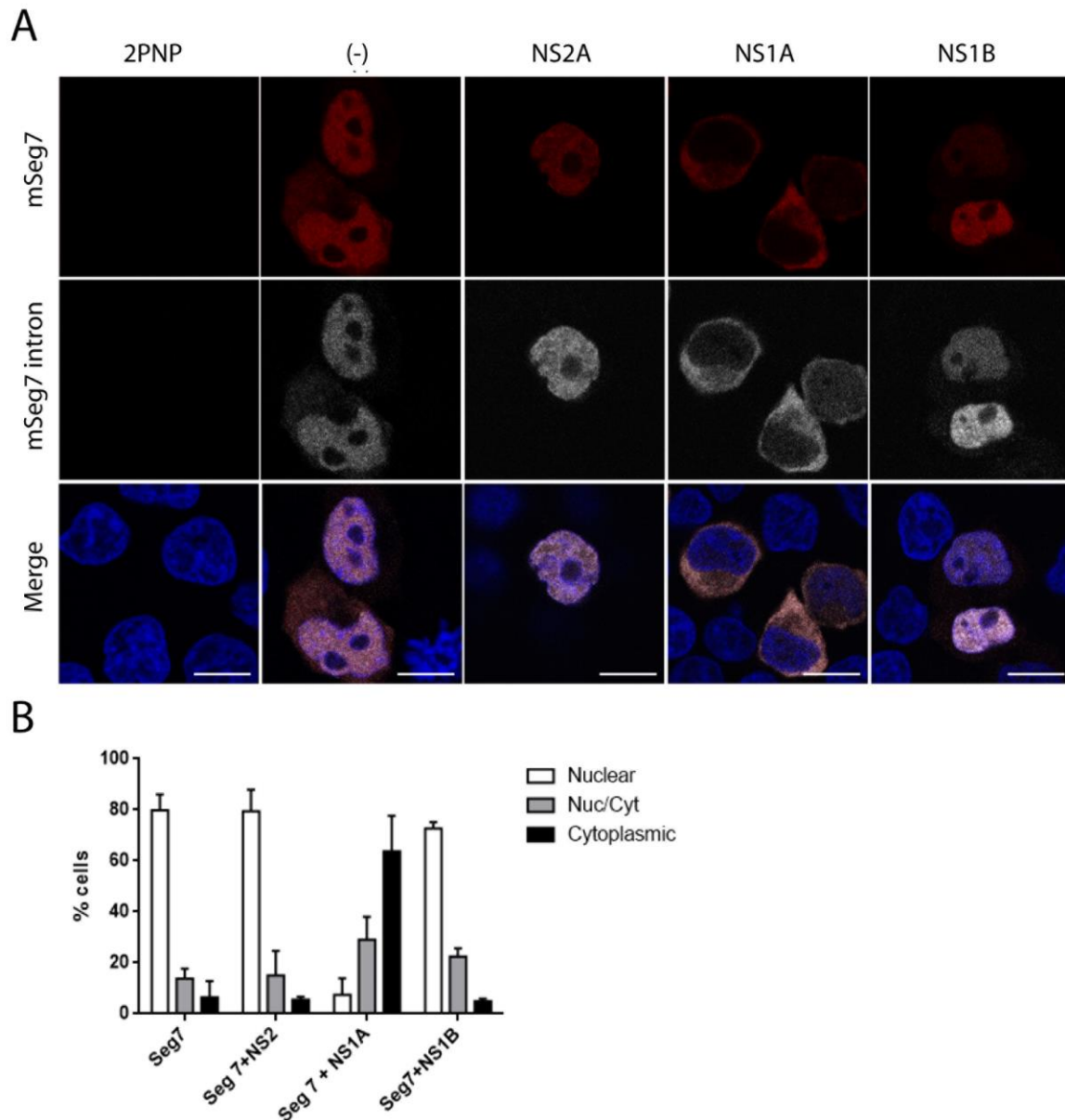


Figure 2.3 Testing the effect of NS1 and NS2 on segment 7 mRNA localisation. A) 293T cells were co-transfected with plasmids expressing each of the influenza polymerase proteins and NP protein (3PNP), or plasmids expressing PB1, PA and NP protein (2PNP), along with a plasmid expressing an artificial segment 7 vRNA. Additional plasmids expressing influenza A NS2 and NS1 (NS2A and NS1A, respectively) proteins or influenza B NS1 (NS1B) protein were added where indicated. Cells were incubated for 24 h and subsequently processed for FISH analysis using Cy3 labelled RNA probes for positive sense segment 7 RNAs (red) and Cy5 labelled probes for the intron sequence of segment 7 (grey). Images were captured using a Leica-TCS confocal microscope and Leica TCS analysis software. Scale-bar indicates 10 μ m. B) Cells expressing segment 7 mRNA were counted and scored according to the cellular localization of this mRNA: predominantly nuclear, nuclear and cytoplasmic (nuc/cyt) or predominantly cytoplasmic. A minimum of 100 cells were scored for each sample. Error bars represent the mean \pm SEM of at least 3 independent experiments. The data for segment 7 without any further additions corresponds to the data shown before in Figure 2.2.

The mRNA localisation phenotypes observed with the intron-specific probe (grey staining) were indiscernible from the patterns obtained when using a probe able to detect all mRNA forms (**Figure 2.3A**). M1 mRNA was retained in the nucleus when segment 7 was on its own, with NS2A and NS1B, but released to the cytoplasm in the presence of NS1A (**Figure 2.3A**). These results indicated that NS1A protein is the viral factor required for the efficient nuclear export of M1 mRNA, specifically.

To put these observations on a more quantitative footing, cells were also scored according to segment 7 mRNA localisation upon the addition of either NS1A, NS2A or NS1B. On its own, or together with NS2A or NS1B, segment 7 mRNA was detected in the nucleus of 70-80 % of cells scored, in the cytoplasm of ~5% of cells and the remainder showed mRNA staining in both cellular compartments (**Figure 2.3B**). In the presence of NS1A, the shift in segment 7 mRNA localisation was very clear across the population of cells. More than 60% of cells showed cytoplasmic staining and nuclear-only staining was observed in less than 10% of scored cells. This quantification strengthened the observations made under the microscope and showed a very significant shift in segment 7 mRNA localisation in the presence of NS1A protein.

Taken together these results indicated that NS1A protein is required for segment 7 mRNA export, in particular M1 mRNA, using an orthomyxovirus species-specific mechanism.

2.2.4 Protein expression analysis in the context of the minireplicon assay

Nuclear export of mRNA is a pre-requisite for protein synthesis since the overwhelming majority of *bona fide* translation occurs in the cytoplasm (Baboo and Cook, 2014; Dolan et al., 2010). Hence, any correlation between segment 7 M1 and M2 protein levels and the segment mRNA export was investigated. 293T cells were transfected with the basic segment 7 RNP set together with other viral components, separately added. Total cell lysates were harvested and analysed by western blotting 24 hours post-transfection (h p.t.). NP levels were comparable in all samples, indicating reasonable transfection efficiencies, while tubulin levels were similar, confirming equal gel loading (**Figure 2.4**). The expression of NS1A, NS2A and NS2B was confirmed by their detection in the expected lanes (**Figure 2.4**, lanes 5-8; note the V5 tag on the NS1A and NS1B proteins). M1 and M2 proteins were not detected in the negative control 2PNP as expected, since segment 7 mRNA synthesis was blocked due to the missing PB2 gene (lane 1). M1 and M2 were detected in all other samples, although not to the same extent for the different conditions. The addition of segments 4 and 6

did not impact on M1 and M2 expression, showing similar levels to segment 7 on its own (lanes 2-4). NS2A presence however, resulted in decreased expression of M1 and M2 proteins (lane 7). M1 and M2 protein expression levels were clearly increased in the presence of segment 8 (which produces both NS1 and NS2) as well as NS1A protein (lanes 5 and 6). This increase in M1 and M2 accumulation in the presence of NS1 was consistent with the more efficient release of segment 7 mRNA to the cytoplasm seen by FISH, as well as with the ability of NS1 to increase translation of viral mRNAs (Enami et al., 1994). However, NS1B also notably increased expression of the segment 7 polypeptides (lane 8), despite its lack of effect on mRNA localisation.

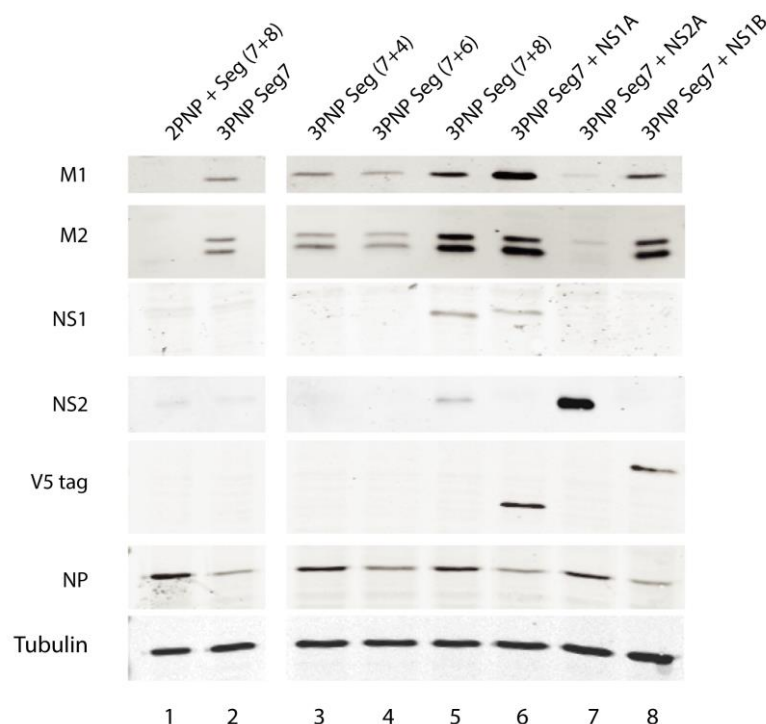


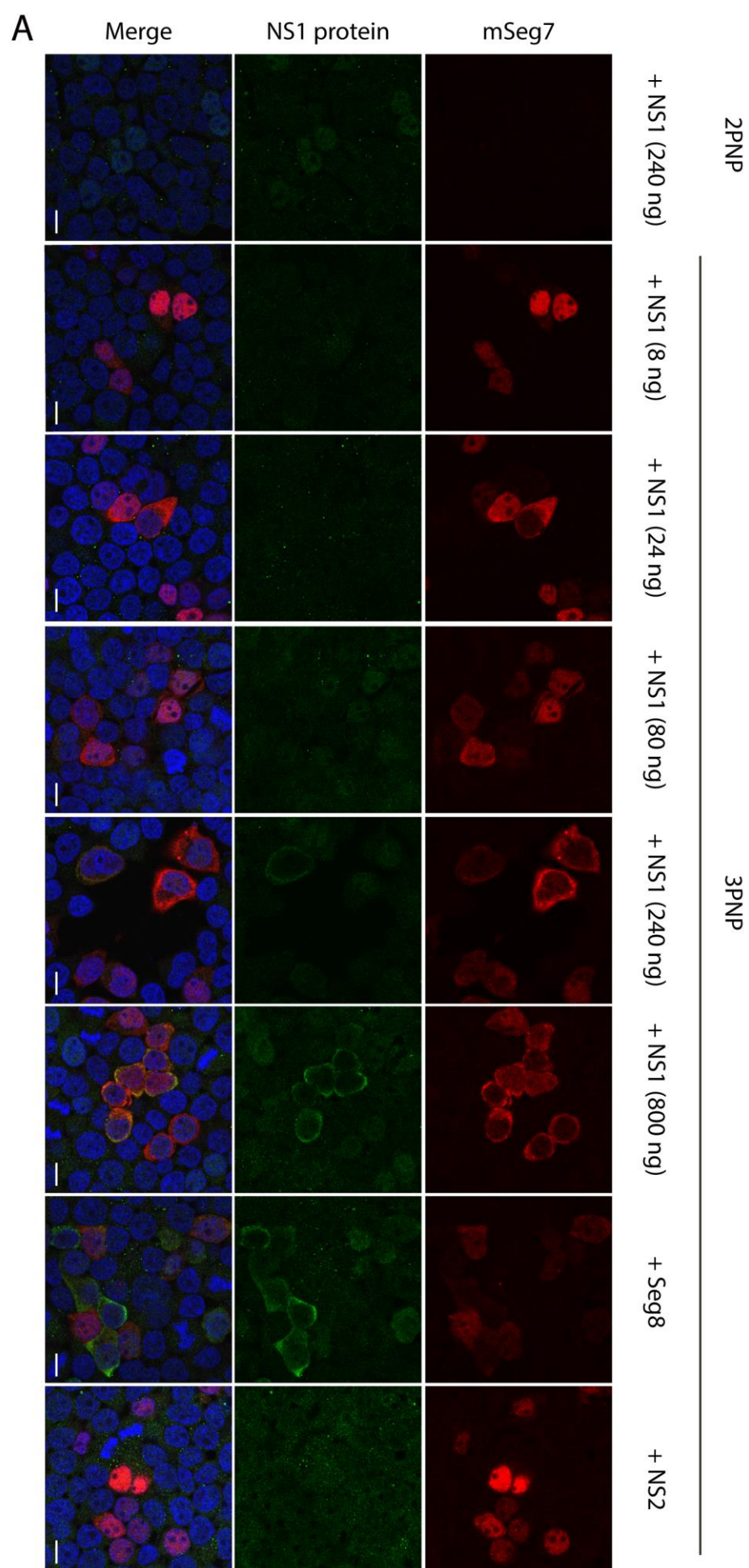
Figure 2.4 Viral protein expression in RNP reconstitution assays. 293T cells were co-transfected with plasmids expressing each of the influenza polymerase proteins and NP protein (3PNP) or plasmids expressing PB1, PA and NP proteins (2PNP) alongside plasmids expressing vRNAs of the indicated segments. 24 h.p.t. cell lysates were harvested in Laemmli's sample buffer and analysed by SDS-PAGE and western blotting for the indicated proteins. Western blots were imaged using a Licor Biosciences imaging platform and associated Odyssey v3.0 software. Results are representative of at least three independent experiments.

2.2.5 Increased levels of NS1A protein expression lead to higher segment 7 mRNA export efficiency and M1 protein expression levels

In order to further establish NS1A protein as the viral factor required for efficient nuclear export of segment 7 mRNA, the effect of increasing the amounts of NS1A protein on mRNA localisation were tested. 293T cells were transfected with the basic segment 7

minireplicon set and increasing amounts of NS1A-expressing plasmid, ranging from 8 to 800 ng. As further controls, additional samples had either segment 8 or NS2 only transfected. Segment 7 mRNA localisation was then analysed by FISH and NS1 expression by indirect immunofluorescence (IF) (**Figure 2.5A**). Red FISH signal corresponding to mRNA could not be detected in the negative control 2PNP and was found in the cytoplasm upon addition of segment 8 and retained in the nucleus in the presence of NS2A protein. Increasing the amounts of NS1A protein resulted in a clear gradual shift of segment 7 mRNA localisation. The mRNA was markedly retained in the cell nucleus when only 8 ng of the plasmid were added and became increasingly more cytoplasmic with increasing amounts of NS1A. When 800 ng of NS1A was added, transfected cells displayed segment 7 mRNA mainly in the cytoplasm (**Figure 2.5A**). Even though the IF signal (in green) obtained with our NS1A serum was not very strong, NS1A protein could be detected when 80 ng of NS1 plasmid was added and the signal became more evident with higher amounts of NS1A. The fact that there was green staining detected also in the presence of segment 8, but not NS2 showed that the antibody used was specific to NS1A (**Figure 2.5A**).

Alongside the mRNA localisation assessed by FISH, protein expression levels were also analysed to confirm the gradual increase of NS1A protein levels as well as to observe the effect of such an increase on M1 and M2 expression levels. 293T cell lysates were harvested for each sample and western blotting analysis was carried out (**Figure 2.5B**). All samples showed comparable levels of NP and tubulin, confirming similar transfection efficiency and equal gel loading for each condition, respectively. The continued increase of NS1A protein expression was notable with the corresponding band becoming faintly but increasingly visible from 80 ng of plasmid transfected onwards. NS1A was also detected in the presence of segment 8 (note that the V5 tag accounts for the size difference between the protein produced by the NS1A expression vector and the untagged NS1A produced from segment 8) but not the NS2A protein used as controls for this experiment (lanes 7-8). M1 and M2 proteins were not detected in the 2PNP negative control lacking PB2 but were otherwise present in all samples. M1 levels were substantially higher in the presence of even the lowest amount of NS1A and then generally increased along with increasing amounts of NS1A. M2 accumulation was also increased by the lowest amount of NS1A plasmid tested, but thereafter levels showed, if anything, a negative correlation with NS1 amount. Overall, these results showed a positive correlation between higher amounts of NS1A and more efficient segment 7 mRNA nuclear export, reflected in higher levels of M1 expression.



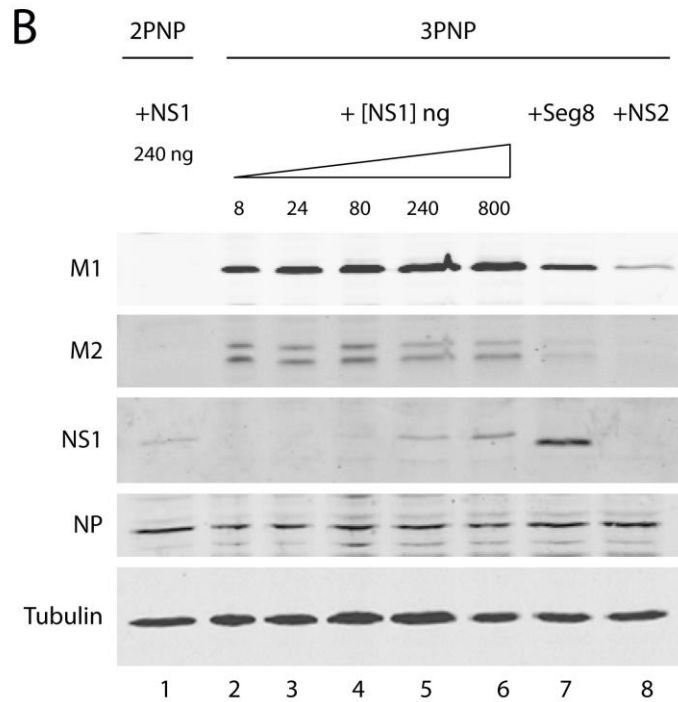


Figure 2.5 Titration of NS1 protein and its effect on the localisation of segment 7 mRNA. 293T cells were co-transfected with plasmids expressing each of the influenza polymerase proteins and NP protein (3PNP), or plasmids expressing PB1, PA and NP protein (2PNP), along with a plasmid to express segment 7 vRNA. Further plasmids expressing either segment 8 vRNA (seg 8), NS2 protein or different amounts of NS1A-expressing plasmid were added as indicated. A) Cells were incubated and 24 h subsequently processed for FISH analysis using Cy3 labelled RNA probes for positive sense segment 7 RNAs (red) and counterstained for NS1 protein (green). Images were captured using a Leica-TCS confocal microscope and Leica TCS analysis software. Scale-bar indicates 10 μ m. B) At 24 h.p.t. cell lysates were harvested and subsequently analysed by SDS-PAGE followed by western blotting for the proteins indicated. Western blots were imaged using a Licor Biosciences imaging platform and associated Odyssey v3.0 software.

2.2.6 NS1 and NS2 protein export activities are not altered by the addition of an EGFP tag

With the aim of expanding the studies regarding the role of NS1A protein on viral mRNA export, the development of an enhanced green fluorescent protein (EGFP)-tagged form of NS1 was explored. This had the aim of solving the problem that our NS1A serum did not give a very strong signal in IF after FISH, as well as providing an affinity “hook” for biochemical assays. An EGFP-tagged NS2A construct was also produced to be used as a negative control. These constructs were produced by conventional PCR-cloning and then tested by transfecting them, separately, with the segment 7 minireplicon set in 293T cells to check for their expression and their effect on segment 7 mRNA localisation by FISH (**Figure 2.6**). EGFP expression was confirmed (green staining) in cells transfected with EGFP alone or with either NS1 or NS2 EGFP-tagged constructs while no green signal was detected in the 2PNP negative control omitting PB2. EGFP and NS2-EGFP displayed a predominantly

nuclear localisation while NS1-EGFP expression was found both in the nucleus as well as more prominently in the cytoplasm. No red staining corresponding to segment 7 mRNA was detected in the negative control and the mRNA was largely nuclear when segment 7 was on its own and transfected with either EGFP alone or NS2A-EGFP. When NS1A-EGFP was added however, the mRNA displayed a clear cytoplasmic localisation (**Figure 2.6**). These results showed that the EGFP tag did not obviously alter the phenotype obtained with the addition of untagged NS2A and NS1A proteins (**Figure 2.3**) and therefore did not mask the previous results. These constructs were thus used in many of the experiments that follow throughout this dissertation. Western blot validation that they produced polypeptides of the expected sizes is shown in the next chapter.

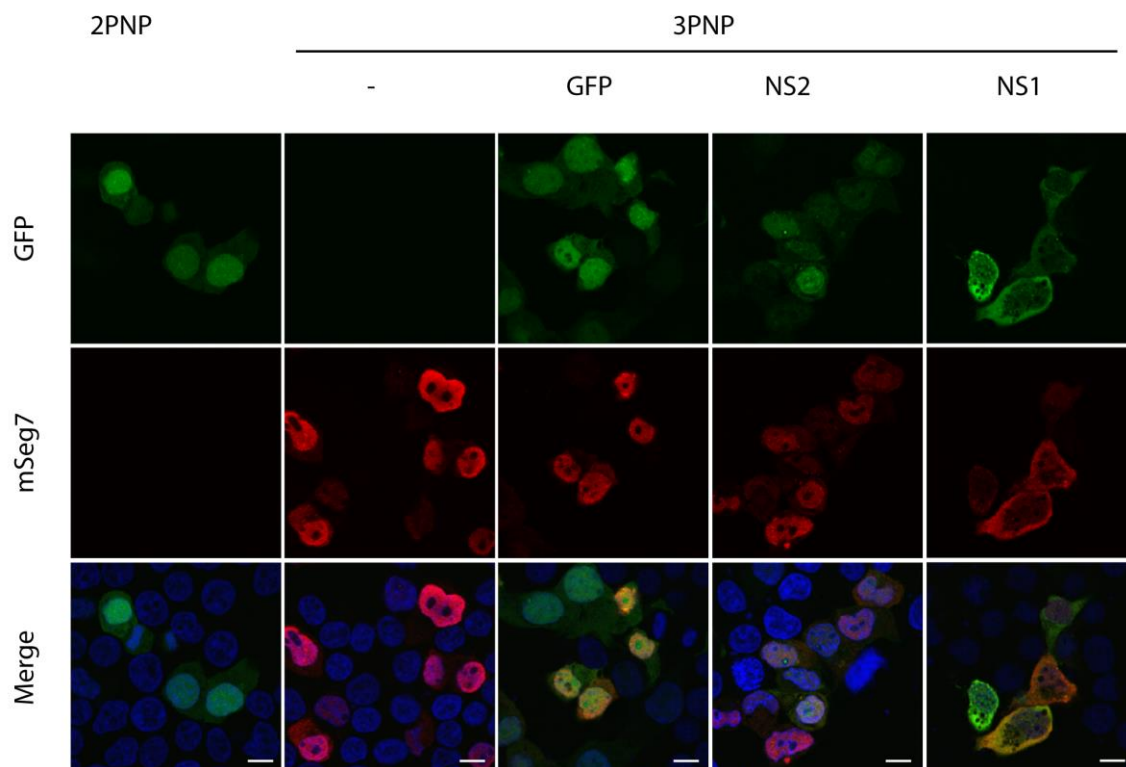


Figure 2.6 Testing the effect of EGFP-tagged NS2A and NS1A proteins on segment 7 mRNA nuclear export. 293T cells were co-transfected with plasmids expressing each of the influenza polymerase proteins and NP protein (3PNP), or plasmids expressing PB1, PA and NP protein (2PNP), alongside an artificial segment 7 vRNA-expressing plasmid. Further plasmids expressing either EGFP, NS1-EGFP or NS2-EGFP were added as indicated. At 24 h.p.t. cells were processed for FISH analysis using Cy3 labelled RNA probes for positive sense viral RNAs of segment 7 (red) and counterstained with DAPI (blue) before imaging for Cy3, DAPI and EGFP (green). Images were captured using a Leica-TCS confocal microscope and Leica TCS analysis software. Scale-bar indicates 10 μ m.

2.2.7 NS1A does not override the negative effect of a poly(U) signal

Data shown so far suggested that NS1A protein is required for the efficient nuclear export of segment 7 mRNA and the question that naturally followed was therefore whether it was sufficient. There is strong evidence suggesting the use of cellular machinery to accomplish nuclear export for the majority of IAV mRNAs (Amorim et al., 2007; Hao et al., 2008; Larsen et al., 2014; Mor et al., 2016; Read and Digard, 2010; Wang et al., 2008). Viral mRNAs are structurally similar to cellular mRNAs in that they both possess a 5' cap structure and a 3' poly(A) tail. However, the mechanisms by which both types of mRNAs acquire these structures differ substantially. Viral mRNAs acquire a 5' cap structure using a cap-snatching mechanism (Bouloy et al., 1979; Bouloy et al., 1978; Krug et al., 1979; Plotch et al., 1979) and their poly(A) tails are produced by the stuttering of the viral polymerase on a poly(U) stretch at the 5' end of the vRNA at the time of transcription (Poon et al., 2000; Poon et al., 1998; Robertson et al., 1981). The poly(A) tails on IAV NA and NP mRNAs have been shown to be required for efficient mRNA nuclear export, as their substitution by a poly(U) tail through the mutation of the vRNA 5' end poly(U) stretch resulted in a substantial decrease in cytoplasmic mRNA (Poon et al., 2000; Read, 2010). Furthermore, NS1 has been demonstrated to interact with poly(A)-binding protein II (PABPII) as well as with CPSF (Chen et al., 1999; Nemeroff et al., 1998) and to viral mRNAs (Wang et al., 2008). It was therefore possible to conjecture that NS1 could facilitate segment 7 mRNA nuclear export by interacting directly or indirectly with its poly(A) tail or poly(A) tail-interacting proteins. Hence, the potential ability of NS1 to override the lack of a poly(A) signal and maintain its capability of supporting mRNA nuclear export of a segment 7 transcript containing a poly(U) instead of a poly(A) tail was assessed. To this end, a mutated segment 7 vRNA containing a poly(A) instead of a poly(U) stretch was used. The stuttering of the viral polymerase on the stretch of this construct would be expected to result in the production of a poly(U)-tailed mRNA. 293T cells were transfected with the mutant segment 7 vRNA-like template (7polyU) in a minireplicon assay in the presence or absence of NS1A-EGFP and the cellular localisation of this poly(U) mRNA was analysed by FISH (**Figure 2.7A**). In the absence of NS1A-EGFP, segment 7polyU mRNA (stained in purple) was retained in the nucleus as expected. However, when NS1A-EGFP was added the mRNA remained markedly nuclear. NP protein staining (in red) showed no differences in localisation in the presence or absence of NS1A protein, while GFP fluorescence confirmed expression of the NS1A fusion protein in cells that also contained active viral RNPs. Cells were scored according to the cellular localisation of the mRNA as before (**Figure 2.7B**). In the absence of NS1A-EGFP, ~85% of cells had segment 7polyU mRNA retained in the nucleus, similar to the ~80% nuclear

retention observed with a poly(A)-containing mRNA in the same conditions. Contrary to what was observed with a poly(A) segment 7 mRNA however, addition of NS1A-EGFP to a segment 7polyU sample did not result in a clear shift of mRNA localisation. In the presence of NS1-EGFP ~65% of cells had the mRNA in the nucleus, though the percentage of cells displaying a cytoplasmic localisation (20%) was higher than when no NS1A-EGFP was added. These results suggested that even though NS1A was required for the efficient export of segment 7 mRNA it was not sufficient, which is in line with studies showing that cellular factors play a role in viral mRNA nuclear export (Amorim et al., 2007; Mor et al., 2016; Read and Digard, 2010; Satterly et al., 2007; Wang et al., 2008).

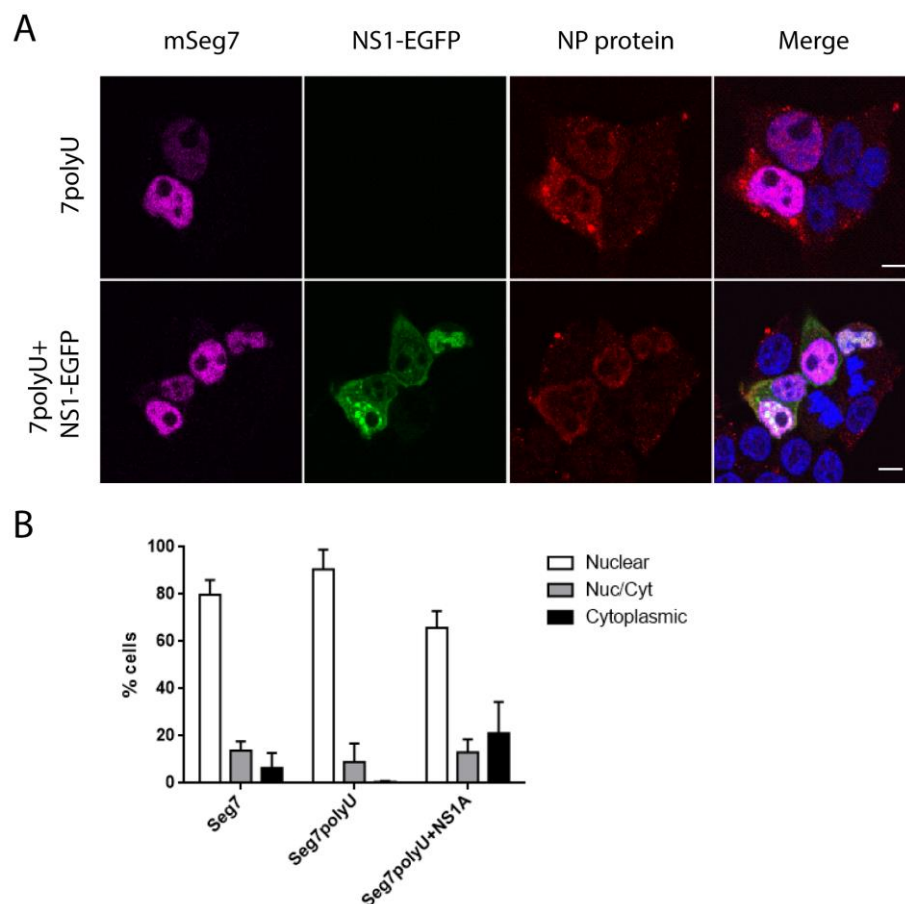


Figure 2.7 Testing whether poly(A) tails are required for influenza A segment 7 mRNA nuclear export. 293T cells were co-transfected with plasmids expressing each of the influenza polymerase proteins and NP protein (3PNP), or plasmids expressing PB1, PA and NP protein (2PNP), alongside a plasmid expressing a mutated segment 7 vRNA analogue with the 5' oligoU sequence replaced with an oligoA sequence (7polyU). A plasmid expressing NS1-EGFP was added where indicated. A) At 24 h.p.t. cells were processed for FISH analysis using cy5 labelled probes for segment 7 positive sense RNA (purple) and counterstained for NP protein (red) and DAPI (blue). NS1-EGFP expression is indicated in green. Images were captured using a Leica-TCS confocal microscope and Leica TCS analysis software. Scale-bar indicates 10 μ m. B) Cells expressing segment 7 mRNA were counted and scored according to the cellular localization of the 7polyU mRNA: nuclear, nuclear and cytoplasmic (nuc/cyt) or cytoplasmic. A minimum of 100 cells were scored for each sample. Error bars represent the mean \pm SEM of at least 3 independent transfections. The data for WT segment 7 without any further additions corresponds to the data shown previously in Figure 2.2.

2.2.8 Effects of NS1A on segment 7 mRNA splicing

The mechanism by which cellular mRNAs achieve nuclear export is closely linked to the mRNA splicing machinery, as splicing factors are recruited to the mRNA via TREX components (Strasser et al., 2002). In influenza A infected cells, NS1 protein has been reported to interfere with cellular mRNA export by blocking the 3' end processing of cellular transcripts (Nemeroff et al., 1998). NS1A has also been implicated in the regulation of segment 7 mRNA splicing in some (but not all) studies (Fortes et al., 1994; Lu et al., 1994; Robb and Fodor, 2012). It was therefore conceivable that, if the M1 and M2 mRNAs have intrinsic differences in transport efficiency, NS1 could indirectly promote segment 7 mRNA export by changing the balance between spliced and unspliced products. To test this hypothesis, 293T cells were transfected with the minireplicon assay basic set of constructs, a segment 7 vRNA template and constructs expressing components of segments 4 and 8 were added separately. At 24 h p.t., cells were harvested, total RNA was extracted and segment 7 mRNA splicing was analysed using radiolabelled primer reverse transcription (**Figure 2.8A**).

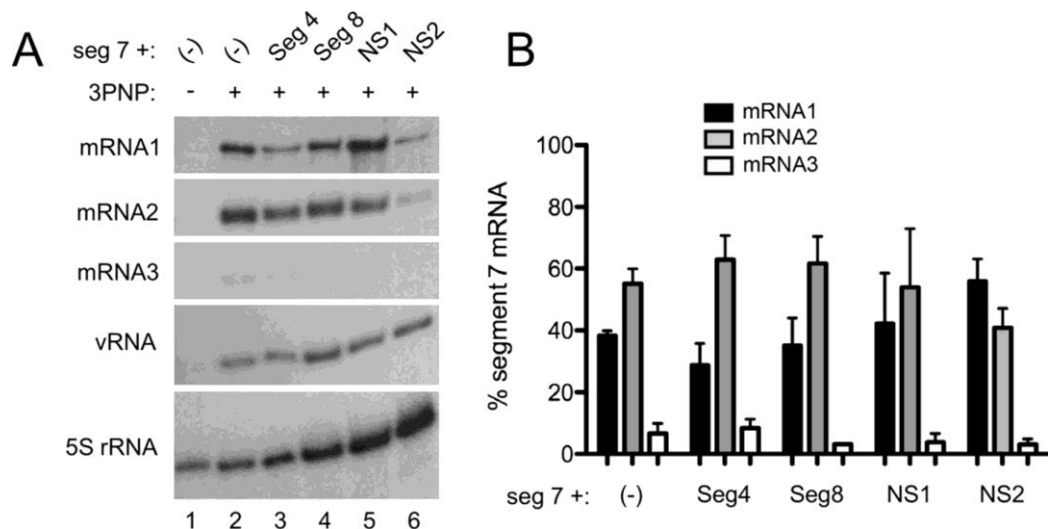


Figure 2.8 Analysis of segment 7 mRNA species produced in RNP reconstitution assays. 293T cells were co-transfected with plasmids expressing each of the IAV polymerase proteins and NP (3PNP: +), or plasmids expressing PB1, PA and NP protein (3PNP: -), alongside a plasmid expressing segment 7 vRNA. Plasmids expressing vRNA from segments 4 and 8 or NS1 or NS2 proteins were added as indicated. A) At 24 h.p.t cells were harvested, RNA extracted and analysed by primer extension using ^{32}P -labelled oligonucleotides for plus or negative sense segment 7 RNAs as well as cellular 5S rRNA as a loading control. Samples were separated by 6% 7M urea-acrylamide gel and visualised by autoradiography. Regions from a single autoradiogram showing products corresponding to the indicated species are shown. B) The intensity of products corresponding to segment 7 mRNAs 1, 2 and 3 was quantified for each sample using ImageJ software. Error bars represent the mean \pm SEM of at least 3 independent experiments.

No segment 7 RNA species were detected in the 2PNP sample which lacked the polymerase subunit PB2 and acted as a negative control (lane 1). Segment 7 vRNA levels were present in similar levels in all 3PNP samples indicating equivalent transfection efficiencies (lanes 2-6). Cellular 5S rRNA presence was also determined to indicate total RNA amounts isolated from each sample. In the RNP reconstitution system, M1 and M2 mRNAs accumulated to approximately equal amounts in the absence of NS1, while mRNA3 formed a minority species (lanes 2, 3 and 6). However, expression of NS1 had variable and generally modest effects on the overall levels of segment 7 splicing; in fact less than NS2, which did not promote cytoplasmic accumulation of the transcripts. Segment 7 mRNA species were also quantified and their ratios determined from replicate experiments (**Figure 2.8B**). M1 mRNA was the most abundant species in all samples (above 50%) except in the presence of NS2 alone, which was the only instance where a shift between the amounts of M1 mRNA and M2 mRNA was observed. The absence of NS1 did not have a substantial effect on the levels of any individual segment mRNA and did not *per se* result in a shift of ratios. Overall, these results indicated that the NS1 protein promotes efficient nuclear export of segment 7 mRNAs without major effects on their differential splicing or overall accumulation.

2.2.9 Effect of blocking cellular splicing on segment 7 mRNA nuclear export

Although the results above suggested that NS1 protein did not promote nuclear export of segment 7 mRNA by skewing the balance of spliced and unspliced segment 7 mRNA species, the involvement of the cellular splicing machinery in nuclear export of this segment's transcripts could not be discarded. Because the mRNAs contain splice donor and acceptor sites, it remained plausible that splicing components could be recruited to the viral mRNA to either facilitate or hinder its export from the nucleus. To address this question, the drug Spliceostatin was used; a compound that inhibits splicing by binding SF3b and blocking spliceosome formation (Kaida et al., 2007). 293T cells were infected at an M.O.I. of 5 with either wild type PR8 virus (WT) or a PR8-NS1 mutant virus (NS1-N81) in which a stop codon was introduced at position 82 and thus is unable to synthesise a full NS1 protein (provided by Dr Helen Wise in the group). Spliceostatin was added (or not) at the time of infection and at 6 h p.i., segment 7 mRNA localisation was observed by FISH, while protein expression and RNA levels were analysed by western blotting and primer extension, respectively (**Figure 2.9**).

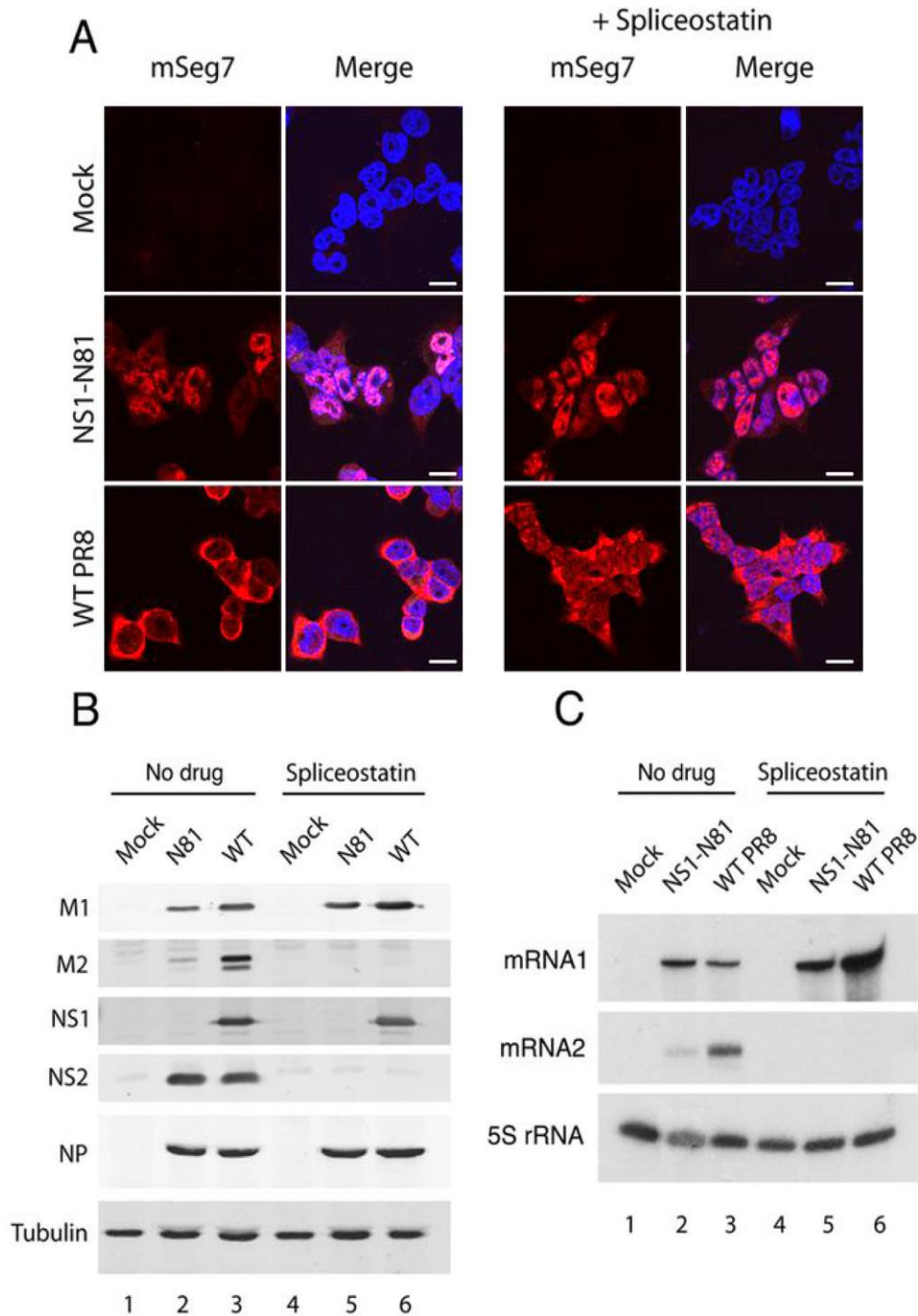


Figure 2.9 Testing the effect of splicing inhibition on segment 7 mRNA export. 293T cells were infected (or mock infected) with either WT PR8 or NS1-N81 at an MOI of 5, and incubated from 1 h p.i. in the absence or presence of 100 μ g/ml Spliceostatin. A. At 6 h.p.i. cells were A) processed for FISH analysis using Cy3 labelled RNA probes for positive sense segment 7 viral RNAs (red). Images were captured using a Leica-TCS confocal microscope and Leica TCS analysis software after counterstaining with DAPI (blue). Scale-bar indicates 10 μ m. B) cell lysates were harvested in Laemmli's sample buffer and subsequently analysed by SDS-PAGE and western blotting for the indicated proteins. Western blots were imaged using a Licor Biosciences imaging platform and associated Odyssey v3.0 software. C) RNA was extracted and analysed by primer extension using a 32 P-labelled oligonucleotide for segment 7 mRNAs 1 and 2 (mM1 and mM2, respectively) as well as cellular 5S rRNA as a loading control. Samples were separated on a 6% 7M urea-acrylamide gel and visualised by autoradiography.

In the absence of Spliceostatin, no mRNA staining was detected in mock infected cells while segment 7 mRNA was in the cytoplasm in WT infected cells as expected. In cells infected with the mutant virus NS1-N81 however, segment 7 mRNA-staining was markedly nuclear (**Figure 2.9A**). The latter observation showed that the lack of an intact NS1 protein resulted in nuclear retention of segment 7 mRNA not only in an RNP reconstitution assay, but also in the context of infection, further validating the role of this protein in promoting segment 7 mRNA nuclear export. When Spliceostatin was added, again no mRNA staining was observed in mock infected cells. Cells infected with WT virus still had the mRNA in the cytoplasm and cells infected with NS1-N81 still showed a clear retention of segment 7 mRNA in the nucleus (**Figure 2.9A**). Hence, phenotypes regarding segment 7 mRNA localisation did not change upon cell infection with either WT or NS1-N81 virus when splicing was inhibited. In order to make sure that the Spliceostatin treatment had worked and inhibited splicing, viral protein expression was analysed by western blotting. Mock infected cells were negative for viral proteins (**Figure 2.9B**, lanes 1 and 4) and loading amounts were checked through tubulin expression levels which were comparable between all samples (lanes 1-6). Full-length NS1 protein was not detected in NS1-N81 infected cells, showing that this mutant virus was indeed NS1-deficient (lanes 2 and 5). NS1 was however detected in WT infected cells in the absence or presence of the drug, to similar levels (lanes 3 and 6). M1 protein was detected in all infected samples and expression levels were higher in WT infected cells than in NS1-81 infected cells, irrespective of the drug being present or not (lanes 2, 3, 5 and 6). M2 protein (produced from a spliced segment 7 mRNA) was present in cells infected with WT and NS1-N81 (lanes 2 and 3) with expression levels being higher upon WT infection. In the presence of Spliceostatin however, M2 protein was no longer detected, consistent with splicing inhibition (lanes 5 and 6). Expression levels of NS2 protein, (also synthesised from a spliced mRNA), further confirmed that the drug had worked, since this protein was detected in the absence of Spliceostatin but not in its presence (lanes 2, 3, 5 and 6). Furthermore, analysis of NS2 protein expression showed that even though NS1-N81 mutant virus was incapable of producing full-length NS1, synthesis of NS2 was not compromised, with equivalent amounts produced by both viruses (lanes 2 and 3). NP protein is produced from an intronless mRNA and was present in equivalent amounts in the presence or absence of Spliceostatin as well as upon infection with WT and NS1-N81 (lanes 2, 3, 5 and 6). The loss of expression of proteins produced from spliced mRNAs was a good but indirect indicator that Spliceostatin had worked. Therefore, in order to determine directly which mRNAs were being produced, radiolabelled primer reverse transcription analyses were performed. Gel loading was certified as comparable between all samples through the inclusion of a primer specific for cellular 5S

rRNA (**Figure 2.9C**). Mock infected samples were negative for the presence of viral mRNAs (lanes 1 and 4). Unspliced M1 mRNA was detected in samples infected with both WT and NS1-N81 and the levels of this mRNA were higher in the presence of Spliceostatin (lanes 2, 3, 5 and 6). Spliced M2 mRNA however, was only detectable in the absence of the drug; confirming that splicing was inhibited by the addition of Spliceostatin (lanes 5 and 6). Overall, these results further indicated that nuclear export of segment 7 mRNA occurred in a splicing-independent fashion. In addition, the data also supported the role of NS1 protein in segment 7 mRNA nuclear export in an infection context.

2.3 Discussion

The aim of the work presented in this chapter was to determine whether specific IAV proteins played a role in viral mRNA nuclear export. IAV synthesizes a relatively small number of polypeptides and thus utilises the host machinery for many processes of its life cycle. Nevertheless, its proteins have a broad spectrum of functions and are not limited to structural purposes. They can be the main contributor in a viral life cycle step, as is the case for viral replication and transcription which is accomplished by the viral polymerase (Neumann et al., 2004) or they can have a more indirect role and act as a bridge between viral components and the host cell machinery. For instance, recruitment of various cellular pathways via viral proteins has been shown for IAV; for example, in the nuclear export of the viral genome (Elton et al., 2001; Martin and Helenius, 1991; Neumann et al., 2000; O'Neill et al., 1998) as well as in cytosolic vRNP trafficking (Amorim et al., 2011; Einfeld et al., 2011; Momose et al., 2011). The cellular NXF1 pathway used to export the bulk of cellular mRNA (Herold et al., 2001) has been shown to be required for IAV mRNA export (Larsen et al., 2014; Read and Digard, 2010). However, how the individual species of viral mRNA are recruited to this pathway remains unclear. Other viruses like HIV and herpesviruses have been shown to use viral proteins as adapters to feed viral mRNAs into the necessary cellular pathways in order to achieve nuclear export (Boyne et al., 2010; Chen et al., 2002; Colgan et al., 2009; Cullen, 2003; Sandri-Goldin, 1998). Hence it was reasonable to postulate that IAV mRNA nuclear export could be achieved (at least in part) with the participation of a viral protein. This hypothesis gained sustenance from an observation made in the Digard laboratory prior to the commencement of this work (recapitulated in **Figure 2.1**) in which segment 7 mRNA was found to be retained in the cell nucleus in an RNP reconstitution assay (Read, 2010). Since the mRNA was cytoplasmic in infected cells (Amorim et al., 2007; Larsen et al., 2014; Mor et al., 2016; Read and Digard, 2010) it was plausible that a viral component that

was absent from the RNP reconstitution assay was necessary for efficient segment 7 mRNA nuclear export. The results presented in this chapter validated this hypothesis and revealed NS1 protein as this viral factor. In addition to increasing our understanding of viral mRNA export the results of this chapter also suggest a novel function of the NS1 protein.

The approach used to determine the viral requirements for segment 7 mRNA export was based on the observation of its localisation in an RNP reconstitution context upon the presence or absence of a particular viral component. The role of NS1 protein in promoting efficient viral mRNA export was suggested by the observation of a clear shift in mRNA localisation from the nucleus to the cytoplasm achieved solely by the introduction of this polypeptide in the RNP reconstitution assay (**Figure 2.3**). These data do not rule out the involvement of viral proteins that are part of the RNP (PB2, PB1, PA and NP or 3PNP) in segment 7 transcript nuclear export, but together they were clearly not sufficient. The same reasoning applies to the viral accessory polypeptides that are synthesised from the 3PNP segments: PB1-F2, PB1-N40, PA-X, PA-N155 (Chen et al., 2001; Jagger et al., 2012; Muramoto et al., 2013; Wise et al., 2009). The capability of promoting viral mRNA export was specific to the NS1 protein since it was the only addition that resulted in the shift of segment 7 mRNA from the nucleus to the cytoplasm. HA and NA are expressed at later times in infection (Palese and Shaw, 2007) and thus were perhaps unlikely candidates to be involved in mRNA export. Nevertheless, the addition of these proteins was also tested and neither found to be able to rescue segment 7 mRNA from the confines of the nucleus (**Figure 2.2**). There is the caveat however, that expression of neither polypeptide was confirmed. The data also suggested that the mechanism by which viral mRNA export is achieved might be specific at the level of individual orthomyxovirus species, since addition of NS1B to the minireplicon assay did not promote segment 7 mRNA export (**Figure 2.3**). It is however possible that NS1B could promote the export of influenza B viral mRNAs. In fact, both NS1A and NS1B have previously been suggested to have the potential to play a role in mRNA export (Schneider and Wolff, 2009).

Analysis of viral protein expression in the context of the minireplicon assay revealed points to take into consideration. Surprisingly, the expression of M1 and M2 proteins could be easily detected by western blotting even in samples where FISH suggested the mRNA was largely retained in the nucleus (**Figure 2.4**; lanes 2, 3, 4, 7 and 8). The higher than anticipated levels of M1 and M2 proteins produced in cells with a low level of mRNA nuclear export could perhaps be due to FISH assays detecting nuclear mRNA better than cytoplasmic

transcripts. The occurrence of nuclear translation of viral mRNAs is another possibility which has been reported to occur in IAV infected cells (Dolan et al., 2010). Nevertheless, M1 and M2 protein levels were considerably higher in the presence of NS1 either when the whole segment 8 or just NS1A were added. This was consistent with NS1 promoting mRNA nuclear export as higher amounts of mRNA being transported to the cytoplasm would result in higher protein expression levels.

In addition to the possible factors considered above, it has also been suggested that NS1A can stimulate the translation of viral mRNAs (Burgui et al., 2003; de la Luna et al., 1995; Enami et al., 1994; Park and Katze, 1995). The presence of NS1A protein was shown to result in increased M1 protein expression even though transcription levels were unaltered indicating that NS1A had a post-transcriptional role (Enami et al., 1994). Another study showed that the presence of NS1A not only resulted in increased viral mRNA and protein accumulation, but also in the increase in size of NP-specific polysomes when NP was co-expressed with NS1A, indicating a role at the translational level (de la Luna et al., 1995). Furthermore, NS1 has been shown to interact with the eIF4GI subunit of the eIF4F translation initiation factor and both were shown to interact with poly(A)-binding protein I (PABPI) suggesting that these proteins could form a complex that could potentially promote the recruitment of 43S initiation complexes to viral mRNAs (Burgui et al., 2003). It is however plausible that at least some of the effects of NS1A observed in these studies could be due to the ability of NS1A facilitating mRNA export rather than having a direct role on viral mRNAs translation.

In the system used here, addition of NS1A alone resulted in more M1 being produced than when the whole segment 8 was added. This could be due to the pPolI segment 8 and NS1A plasmids having different expression efficiencies since the backbone vector is not the same and the cytomegalovirus immediate early promoter present in the latter construct is considered a strong expression method. However, expression of NS1 from the complete segment 8 will also be driven by the viral polymerase and in fact western blot analysis showed similar levels of NS1 expression from the two approaches (**Figure 2.4**, lanes 5 and 6). It is also possible that NS2 protein could have an antagonistic effect since the expression of NS2 alone apparently repressed M1 and M2 expression (**Figure 2.4**, lane 7), while the FISH phenotype was also clearer when adding just NS1A as opposed to adding segment 8. The presence or absence of NS2 protein expression in a minireplicon assay that uses segment 7 as viral template is likely to make a considerable difference since M1 and NS2 proteins are used

for vRNA nuclear export (Martin and Helenius, 1991; Neumann et al., 2000; O'Neill et al., 1998). As a consequence, when segment 7 is transfected together with the whole segment 8 that expresses NS1 and NS2, both mRNA and vRNA molecules are exported from the nucleus in the cytoplasm (Amorim et al., 2011). However, when NS1 is present, but not NS2, segment 7 mRNA can be exported but not vRNA. Hence, it is conceivable that when NS2 is present the balance between transcription and replication might be affected by the fact that vRNA export is occurring, possibly further downregulating mRNA production and thus affecting protein expression levels. NS2 protein has in fact been reported to regulate the switch between transcription and replication of IAV by reducing the amounts of mRNA and increasing the accumulation of vRNA. It was suggested that NS2 acted directly on the viral replication machinery since NS2 was shown to affect mRNA and vRNA levels in a virus type specific manner (Bullido et al., 2001; Robb et al., 2009). This NS2-dependent switch from transcription to replication in IAV may reflect regulation by small viral RNAs (svRNAs). These are short molecules comprising 22-27 nucleotides from the 5' end of each viral segment and their expression was associated with a bias in the viral polymerase activity from transcription toward replication. RNP reconstitution assays showed that svRNA expression required the viral polymerase, NP and NS2 proteins (Perez et al., 2010). The reports suggesting a role for NS2 in regulating, directly or indirectly, the switch from transcription to replication are supported by our data and provide with a plausible explanation for the poor M1 and M2 protein expression levels observed in a minireplicon assay where NS2 is present and NS1 is absent (**Figure 2.4**).

The strategy used to make the FISH probes used here could not provide direct evidence for the requirements of M2 mRNA export, but it was possible to show that NS1 promoted the efficient nuclear export of M1 mRNA using an intron specific probe. Since M1 is an unspliced mRNA it was conceivable that the export of this transcript could be interlinked with splicing. NS1 could, in theory, facilitate M1 mRNA export by releasing the mRNA from the cell splicing machinery. The data however, indicated that nuclear export of M1 mRNA occurs via NS1 in a splicing-independent fashion (**Figures 2.8** and **Figure 2.9**). Analysis of the relative ratios of mRNA3, M1 and M2 mRNAs in the context of a minireplicon assay showed that the presence or absence of NS1 protein did not alter substantially the balance of spliced and unspliced transcripts; something that might have been expected if NS1 functioned by removing mRNA otherwise destined for splicing (**Figure 2.8**). In addition, the use of Spliceostatin A which inhibits pre-mRNA splicing by interacting with SF3b, an essential component of the spliceosome and thus preventing its assembly (Kaida et

al., 2007), provided strong evidence that the splicing machinery did not influence segment 7 mRNA export. Spliceostatin A treatment did not change the cytoplasmic localization of segment 7 mRNA in WT infected cells nor the nuclear retention in cells infected with N81, an NS1-deficient virus. At the time this work was performed, NS1 had already been hypothesized as being somehow involved in IAV mRNA nuclear export (Schneider and Wolff, 2009). The discoveries that NS1 could interact both with NXF1, components of the nuclear pore complex (Satterly et al., 2007) and with viral mRNAs (Park and Katze, 1995; Wang et al., 2008) lead to the proposal that IAV could utilise the NXF1/Nxt1 cellular pathway for the export of its mRNAs with the participation of NS1 (Wang et al., 2008). However, there was no direct evidence that indeed NS1 had an effect on the export of viral mRNAs and the mechanism of IAV mRNA nuclear export was poorly understood. After the experimental work described in this dissertation was finalised, but before its submission, one study was published that made a more concrete link between NS1 and segment 7 mRNA export (Mor et al., 2016). In this study, NS1 was found to promote M1 mRNA localisation at nuclear speckles where splicing of segment 7 mRNA was shown to occur. The authors also found TREX components localising to nuclear speckles and proposed that both splicing and initial steps of mRNA export occurred in these cellular structures. The data described in this chapter are in agreement with the Mor *et. al.*, study in finding that NS1 plays a role in segment 7 mRNA export. However, the potential link between nuclear export and mRNA splicing was examined in this dissertation and indicated that NS1 being able to promote efficient nuclear export of segment 7 mRNA did not depend on transcript splicing (**Figure 2.9**).

One of the many functions attributed to NS1 protein (Hale et al., 2008b) is to actually block cellular mRNA nuclear export (Nemeroff et al., 1998; Qian et al., 1994; Qiu and Krug, 1994; Satterly et al., 2007). NS1 has been shown to interact with the 30 KDa subunit of CPSF thereby inhibiting the 3' end processing of cellular mRNAs (Nemeroff et al., 1998). Although viral mRNAs are structurally similar to cellular mRNAs, the acquisition of the 5' cap structure as well as the 3' end poly(A) tail is done differently. Viral mRNAs have a poly(U) stretch in the genome in which the viral polymerase stutters resulting in a poly(A) tail (Neumann et al., 2004) with no involvement of the cellular 3' end mRNA processing. Hence NS1 would have opposite effects on cellular and viral mRNAs which could be a viral regulation mechanism. It has been suggested that inhibition of cellular mRNA export via NS1 would provide with a larger source of 5' cap structures to be scavenged by the viral

polymerase. Using the same reasoning it can be speculated that retention of cellular mRNAs would decrease the competition for the cellular machinery involved in mRNA export.

Taken together, the data presented in this chapter showed that NS1 protein is required for efficient nuclear export of segment 7 mRNA, specifically the M1 transcript. NS1 was shown to be necessary for segment 7 mRNA export both in the context of minireplicon assays and viral infection. Furthermore, the data suggested that the export function of NS1 is specific within the influenza A and B lineages, and discouraged the hypothesis of the cellular splicing machinery being involved in the export mechanism.

CHAPTER 3 – MECHANISTIC INSIGHTS INTO THE ROLE OF NS1 IN SEGMENT 7 mRNA NUCLEAR EXPORT

3.1 Introduction

Various nuclear-replicating viruses have been shown to accomplish export of their transcripts through the recruitment of cellular pathways via specific viral proteins. The HIV-1 regulatory protein Rev has been shown to trans-activate the nuclear export of unspliced mRNAs by targeting specific *cis*-acting RNA sequences – Rev response elements (RRE) in the viral mRNA (Malim et al., 1989). Rev then interacts with CRM1 which targets components of the nuclear pore complex to accomplish mRNA transport to the cytoplasm (Cullen, 2003). Herpes simplex virus (HSV) on the other hand, uses the viral protein ICP27 to bind the mostly intronless viral mRNAs (Sandri-Goldin, 1998) and activates nuclear export through a direct interaction between ICP27 and Aly, acting as a viral homolog of UAP56 and thus introduces viral mRNAs into the cellular NXF1 nuclear export pathway (Chen et al., 2002). The use of the CRM1 pathway has been discounted for IAV mRNA nuclear export since inhibition of this pathway using Leptomycin B did not affect viral gene expression or mRNA cellular localisation (Elton et al., 2001; Larsen et al., 2014; Ma et al., 2001; Read and Digard, 2010; Watanabe et al., 2001). Repression of NXF1 expression by siRNA treatment however, revealed that this cellular protein was necessary for IAV mRNA export although not necessarily to same degree for all the segments (Larsen et al., 2014; Read and Digard, 2010). Furthermore NXF1 has been shown to interact with IAV mRNAs (Satterly et al., 2007; Wang et al., 2008) strengthening the hypothesis that this pathway might be used for viral mRNA export although how the viral transcripts could be recruited to this pathway remains to be elucidated.

The previous chapter provided data showing that the viral protein NS1 is required for the efficient nuclear export of segment 7 mRNA. Hence, the aim of this chapter was to understand the specific role of NS1 in promoting mRNA nuclear export and to test the hypothesis that it provides a link between the viral transcripts and the host export machinery.

3.2 Results

3.2.1 Identification of NS1 functional domains necessary for segment 7 mRNA export in RNP reconstitution assays

As discussed in the previous chapter, NS1 protein was shown to be required for the efficient nuclear export of segment 7 mRNA. A significant number of functions have been attributed to this protein, the main one being to antagonize the interferon response (Hale et al., 2008b). The multifunctional NS1 protein has two functional domains: an N-terminal RNA-binding domain and a C-terminal effector domain to which particular and different roles are ascribed (Hale et al., 2008b). In order to determine whether the segment 7 mRNA export function could be localised specifically to one of the two functional domains of the protein, mutant forms of the protein fused with EGFP were used in the minireplicon system. To study the impact of the effector domain, most of it was deleted by introducing a stop codon at position 82 of NS1. This mutant construct produced a truncated form of NS1 encompassing the first 81 a.a. of the protein and the synthesis of NS2 was not compromised (Zurcher et al., 2000). The same approach could not be used to study the RNA-binding domain of NS1 as deleting the entire domain would interfere with the production of NS2 due to sequence overlap. Instead, a.a. substitutions accepted in the literature as inhibiting this functional domain were used. Since two specific residues, at positions 38 and 41, were identified as necessary for NS1 RNA-binding activity (Wang et al., 1999), both single mutants carrying only one substitution (of the normally positively charged residue to alanine) as well a double mutant carrying both were used. As an additional control, a “gain-of-function” NS1 mutant protein that was not expected to negatively affect nuclear export (NS1-S+I) was added. The mutations present in this polypeptide restored the ability of NS1 to bind CPSF, an interaction that does not exist in the PR8 strain used as template to create the NS1 mutant proteins (Kochs et al., 2007).

293T cells were transfected with plasmids encoding the three viral polymerase subunits (PB2, PB1, PA), NP, a viral template for segment 7 and either EGFP, NS1-EGFP, or a mutant NS1-EGFP. The negative control used in this experiment lacked the PB2 polymerase subunits so transcription and replication of segment 7 would not occur. 24 h p.t. cells were fixed and processed for FISH in order to observe segment 7 mRNA localisation (**Figure 3.1**). As expected, in the negative control lacking PB2 (2PNP), no mRNA staining (red) could be detected while transfection was confirmed by the presence of EGFP (green). When all the

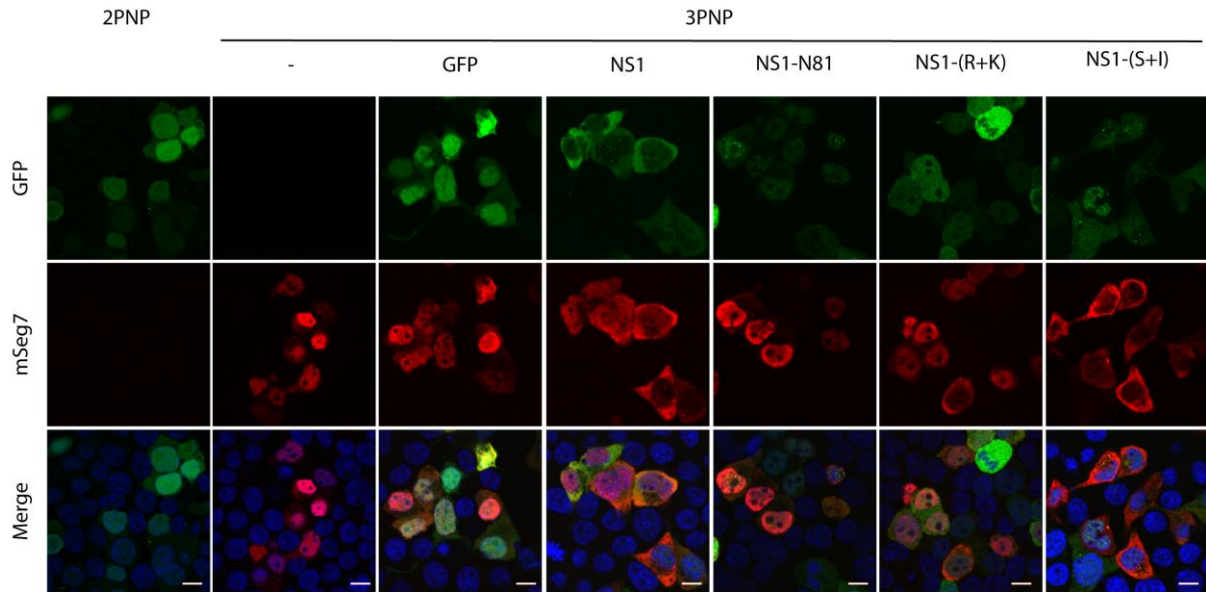


Figure 3.1 Analysis of segment 7 mRNA localisation in the presence of NS1 mutant proteins in transfected cells. 293T cells were co-transfected with plasmids expressing each of the influenza polymerase proteins and NP protein (3PNP) or plasmids expressing PB1, PA and NP proteins (2PNP) alongside plasmids expressing segment 7 vRNA. Plasmids expressing GFP, NS1-GFP or GFP-tagged NS1 mutant protein were added as indicated. Cells were incubated at 37 °C and processed for FISH analysis at 24 h p.t. using Cy3 labelled RNA probes for the detection of positive sense viral RNA from segment 7 (red) and GFP expression was detected by confocal microscopy (green). Images were captured using a Leica-TCS confocal microscope and Leica TCS analysis software. Scale-bar indicates 10 µm.

basic components for RNP reconstitution were present, segment 7 mRNA could be detected predominantly in the cell nucleus and even when EGFP was added, the nuclear retention of the mRNA persisted. In the presence of NS1-EGFP, the shift in mRNA localisation from the nucleus to the cytoplasm was evident, as expected from work shown in the previous chapter. When an NS1-N81 mutant protein which lacked the entire effector domain was used however, the mRNA was again found to be mostly retained in the nucleus, indicating that if this domain was missing, the ability of NS1 to promote mRNA export was lost (**Figure 3.1**). Analysis of the effect of adding an NS1 RNA-binding mutant (NS1-R+K) showed a similar outcome; the mRNA was found mainly in the nucleus, suggesting that a functional N-terminal domain is also required in order for NS1 to be capable of promoting efficient export of segment 7 mRNA (**Figure 3.1**). In the presence of NS1-S+I, segment 7 mRNA was localised largely in the cytoplasm (**Figure 3.1**). This supported the conclusion that the phenotype observed with the addition of NS1-N81 and NS1-R+K were due to the specific mutations introduced. The presence of each NS1 mutant protein was confirmed by EGFP expression (green). All NS1 constructs were found both in the nucleus and the cytoplasm, but the WT polypeptide showed a greater bias towards cytoplasmic localisation than the mutants, especially NS1-N81 where the fluorescence was observed mainly in the cell nucleus. Taken together, these data indicated

that, in order to preserve its mRNA export function, NS1 protein required both functional domains to be present.

3.2.2 Analysis of minireplicon-driven viral protein expression in the presence of mutant NS1 proteins

In the previous chapter it was shown that inclusion of the NS1 protein in a segment 7 RNP reconstitution assay led to more efficient nuclear export of the viral mRNA and to higher expression of the segment-encoded proteins, M1 and M2. Further analysis (above) showed that certain NS1 mutant proteins had lost the ability of promoting mRNA export. To test if the correlation between mRNA export and protein expression efficiency was maintained, their effects on the production of segment 7 gene products was analysed by western blotting of 293T cell lysates collected 24 h.p.t. (**Figure 3.2**). Tubulin levels were assessed to ensure that

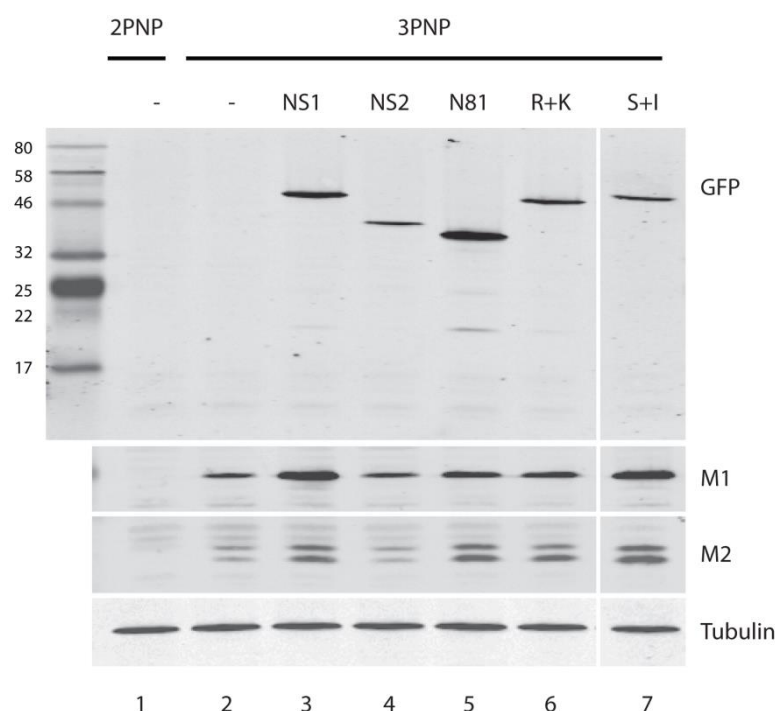


Figure 3.2 Analysis of the effect of NS1 mutations on M1 and M2 expression in the RNP reconstitution assay. 293T cells were co-transfected with plasmids expressing each of the influenza polymerase proteins and NP protein (3PNP) or plasmids expressing PB1, PA and NP proteins (2PNP) alongside a plasmid expressing segment 7 vRNA. Plasmids expressing GFP, NS1-GFP or a GFP-tagged NS1 mutant protein were added as indicated. Cells were incubated at 37 °C and at 24 h p.t., cells were harvested and cell lysates analysed by western blotting for the proteins indicated. A molecular mass marker is included on the left side of the GFP panel with the sizes of each of the bands indicated, to confirm that the GFP-tagged constructs produced proteins of the expected sizes.

the loading was equivalent between all samples. The expression of NS2, NS1 and NS1 mutants was confirmed by blotting using an antibody against EGFP (lanes 2-7). It is to be noted that the size for NS1-N81 was lower since this mutant produces a truncated form of NS1 containing only the first 81 a.a. (lane 5). An RNP-reconstitution negative control that lacked PB2 showed no M1 or M2 expression as expected, since viral transcription and replication were blocked due to the missing polymerase subunit (lane 1). Otherwise, M1 and M2 protein expression was detected in all samples. As seen and discussed previously, M1 and M2 levels were lower in the presence of NS2 (lane 4) as well as in the absence of any additional segment 8 protein (lane 2). When analysing M1 and M2 expression levels according to the NS1 protein present, it was clear that the levels of M1 protein were lower in samples containing NS1 mutants that did not have the ability to export segment 7 mRNA (lanes 5 and 6). Conversely, higher levels of M1 were detected in the sample containing WT NS1 (lane 3) and the positive control NS1 mutant, NS1-S+I (lane 7). Expression of M2, however, did not appear to be decreased in the presence of NS1 export-incompetent proteins (lanes 5 and 6), in fact presenting levels of accumulation similar to WT NS1 and NS1-S+I (lanes 3 and 7). These results are in agreement with the finding discussed previously showing that the presence of NS1 resulted in higher expression levels of M1. Taken together, the data established a correlation between an export-competent NS1 and higher M1 protein expression levels, being in line with the hypothesis that NS1 promoted the efficient nuclear export of M1 mRNA.

3.2.3 The effect of NS1 mutant proteins on segment 7 mRNA localisation in the context of viral infection

In order to validate the effect of NS1 mutations on segment 7 mRNA nuclear export observed in minireplicon assays (**Figure 3.1**), infection experiments were performed using PR8 mutant viruses carrying the same changes in NS1 as the EGFP-tagged constructs used in the transfection-based assays. 293T cells were either mock infected, infected with WT PR8 (WT) or a mutant virus carrying a lesion in the coding region of NS1 (that left the NS2 protein unchanged) at an M.O.I. of 5. Cells were fixed at 6 h.p.i. and processed for FISH analysis to determine the localisation of segment 7 mRNA. Subsequently, cells (including ones from independent replicate experiments) were scored according to the cellular localisation of the mRNA for all samples and the results were quantified in terms of percentage of cells counted. No viral mRNA signal (green) was detected in the mock-infected

sample while DAPI staining (blue) indicated the presence of cells (**Figure 3.3A**). Cells infected with WT virus had segment 7 mRNA in the cytoplasm and cells infected with NS1-N81 presented the viral mRNA retained in the cell nucleus (**Figure 3.3A**), as shown in the previous chapter. Cytoplasmic mRNA localization was evident in approximately 85% of WT infected cells, with the remainder showing segment 7 mRNA in both nucleus and the cytoplasm (**Figure 3.3B**). In cells infected with the NS1-N81 mutant virus, the mRNA nuclear retention phenotype was also clear; mRNA was observed in the cell nucleus in more than 80% of cells scored, in both nucleus and cytoplasm of 15% of cells, and mainly in the

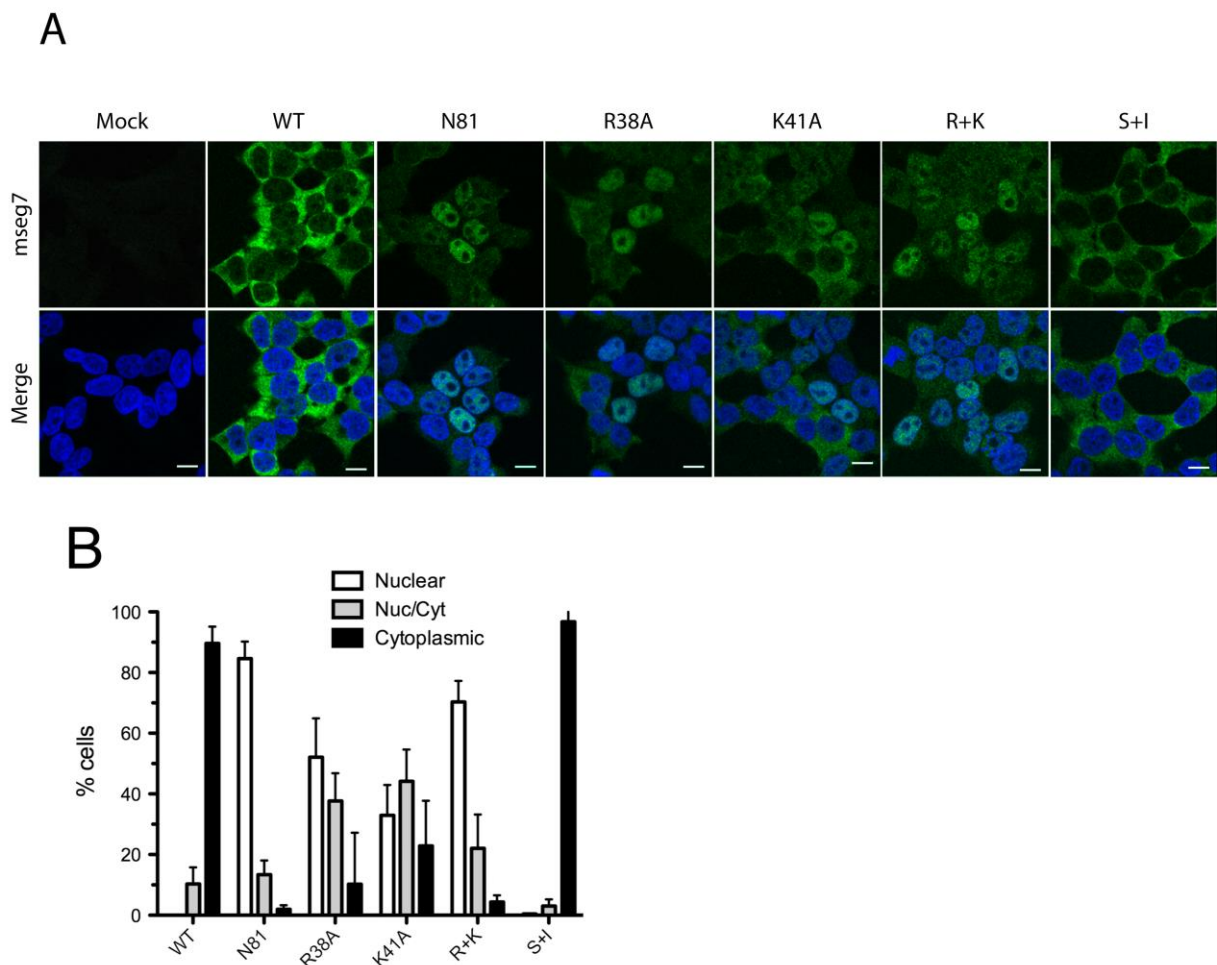


Figure 3.3 Cellular localisation of segment 7 mRNA in cells infected with NS1 mutant viruses. 293T cells were mock infected or infected with the indicated viruses at an M.O.I. of 5. A) At 6 h p.i., cells were processed for FISH and stained for segment 7 mRNA using a specific RNA fluorescent probe (green) and for DNA using DAPI (blue). Images were captured using a Leica-TCS confocal microscope and Leica TCS analysis software. Scale-bar indicates 10 μ m. B) Individual cells were scored according to the predominant cellular localisation of segment 7 mRNA, considering three phenotypes: nuclear, cytoplasmic or mixed. Values are the mean \pm SEM from three to six independent experiments.

cytoplasm of less than 5% of cells. Analysis of the NS1 RNA-binding single mutants (NS1-R38A and NS1-K41A) showed an overall nuclear retention phenotype, as only ~10% and ~20% of cells presented largely cytoplasmic mRNA staining respectively. However, the

percentage of cells with mRNA in both cellular compartments was higher in these samples (~40-50%) than in cells infected with NS1-N81, where the majority of cells scored displayed the mRNA mainly only in the cell nucleus. Cells infected with the RNA-binding domain double mutant (NS1-R+K) showed a phenotype more similar to the NS1-N81 mutant, as segment 7 mRNA was found mainly in the nucleus (~70%) and only ~5% of cells presented cytoplasmic mRNA. Lastly, analysis of the NS1-S+I mutant, used as a control since the change in this mutant virus restored the CPSF binding ability lacking in the WT virus, showed no mRNA nuclear retention, as expected. Infection with this mutant resulted in the vast majority (>95%) of cells displaying viral mRNA in the cytoplasm; the same phenotype observed upon infection with WT. These results provided further validation that the minireplicon system could be used as a reliable tool for the study of viral mRNA transport, since equivalent results were obtained in the two contexts; of infection using NS1 mutant viruses and plasmid-based reconstitution assays. The data also showed that the nuclear retention previously ascribed to the absence of NS1 protein in the context of transfection was maintained upon infection. In addition, the RNA-binding domain single mutants NS1-R38A and NS1-K41A showed an intermediate phenotype compared to the double mutant NS1-R+K.

3.2.4 Protein expression analysis in IAV infected cells where mRNA nuclear export was enabled or inhibited

Analysis of viral protein expression in transfection assays in which the nuclear export of segment 7 mRNA was modulated showed that the production of M1 and to some extent, M2 proteins decreased when NS1 was absent or one of its functional domains was mutated. Next, this correlation between segment 7 mRNA export and protein synthesis, potentially determined by NS1 protein promoting a more efficient mRNA export, was tested in the context of viral infection by examining protein accumulation in infected cell lysates. 293T cells were infected (or mock infected) with either WT PR8 or the NS1 mutant viruses at an M.O.I. of 5. Cell lysates were harvested at 6 h p.i. and analysed by western blotting for M1, M2, NS1 and NP (**Figure 3.4A**). Mock infected cells were used as a negative control and did not show any viral protein expression (lane 1). NP levels were similar in all infected samples confirming comparable levels of infection, while tubulin levels showed that the loading amounts were equivalent in all samples. The presence of NS1 was confirmed in all samples with the exception of mock infected (lane 1) cells. Cells infected with NS1-N81, which produced a truncated form of NS1 not recognised by the antisera used that bound to the C-

terminal domain of the protein, produced a trace of full length NS1 (lane 3), most likely resulting from reversion of the stop codon. M1 and M2 expression was detected in all infected samples but generally lower levels of both proteins were present in samples infected with NS1 mutant viruses that failed to normally export segment 7 mRNA (compare lanes 3-6 with lane 2). Cells infected with the NS1-S+I mutant which allowed mRNA export to occur efficiently showed higher accumulation of M1 and M2 polypeptides compared to WT (lane 7). Quantification of replicate experiments showed that poor nuclear export of segment 7 mRNA corresponded to around a 3- to 5-fold reduction in the quantity of M1 relative to that of NP, and 2- to 3-fold reductions in the amount of M2 (**Figure 3.4B**). These results from viral infections are in line with the ones obtained using the minireplicon assay (**Figure 3.2**) and further validated the correlation observed between NS1-promoted mRNA export and increased levels of M1.

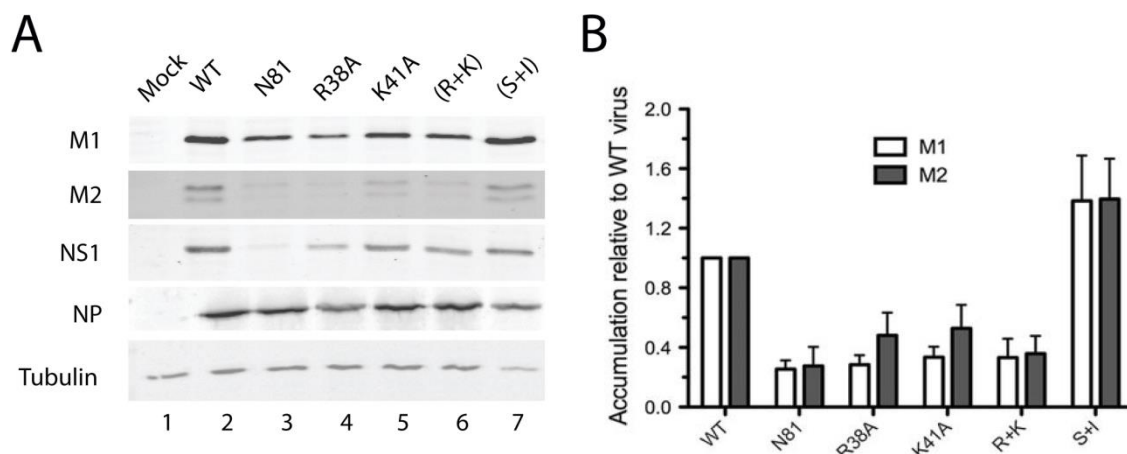


Figure 3.4 Analysis of viral protein expression in cells infected with NS1 mutant viruses. 293T cells were mock infected or infected with the indicated viruses at an M.O.I. of 5. A) At 6 h p.i., cells were harvested and lysed for western blotting analysis for the indicated polypeptides. B) M1 and M2 accumulation from replicate experiments were quantified and expressed as a ratio relative to NP expression. Values plotted are normalised to the ratio seen with WT virus and correspond to the mean \pm SEM of three independent experiments.

3.2.5 Ability of mutant NS1 proteins incapable of promoting efficient segment 7 mRNA nuclear export to interact with cellular protein NXF1

The use of the cellular NXF1 export pathway by IAV to transport its mRNAs from the host cell nucleus to the cytoplasm has been proposed in multiple studies (Hao et al., 2008; Larsen et al., 2014; Read and Digard, 2010; Wang et al., 2008). NS1 protein has also been hypothesised to play a role in viral mRNA nuclear export, since its interaction with NXF1

was demonstrated previously (Larsen et al., 2014; Satterly et al., 2007; Wang et al., 2008). It was then relevant in the context of this study to verify that the interaction between NXF1 and NS1 existed under the specific conditions used here. Furthermore, since certain mutant NS1 proteins were found to be unable to promote efficient nuclear export of segment 7 mRNA, it was also important to test if any of the export-incompetent NS1 mutants also failed to interact with NXF1.

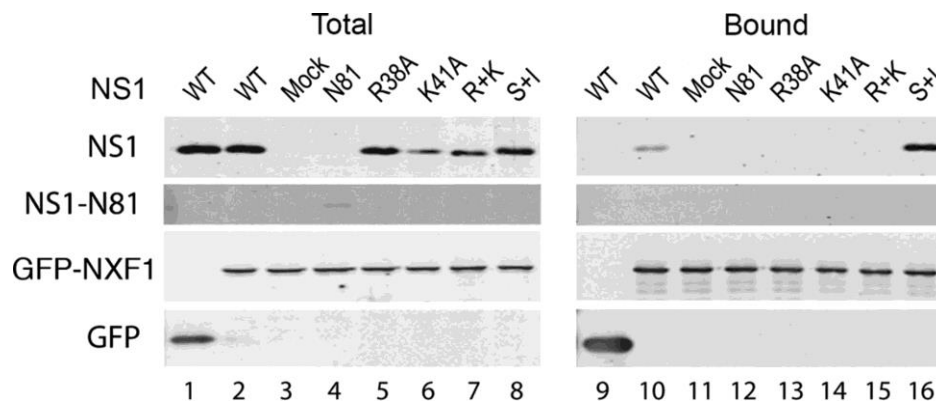


Figure 3.5 Interaction between NXF1 and NS1. 293T cells were transfected with plasmids encoding GFP or GFP-NXF1 and 48 h later either mock infected or infected with the indicated NS1 mutant viruses at an M.O.I. of 5. Cells were harvested at 6 h p.i. and cell lysates were examined by western blotting for the indicated proteins before (Total) or after (Bound) being subjected to GFP-Trap pulldown assays.

To test this, 293T cells were transfected with either GFP or GFP-NXF1 and 48 h later mock infected or infected with the WT or the various NS1 mutant viruses at an M.O.I. of 5. At 6 h p.i. cells were collected and lysed, and the supernatants were subjected to GFP-Trap pulldown. Western blot analyses of total and bound fractions showed that GFP-NXF1 and GFP were expressed as expected and bound well to the affinity matrix (**Figure 3.5**). NS1 was present in all infected samples, including that with the truncated N81 protein, although detection of this last polypeptide required use of an RNA-binding domain-specific antiserum and was inefficient (lanes 1 and 2 and lanes 4 to 8). Consistent with previous reports (Larsen et al., 2014; Satterly et al., 2007; Wang et al., 2008), WT NS1 co-precipitated with NXF1, as did the NS1 S+I mutant (lanes 10 and 16). This binding was specific, since no interaction of WT NS1 with GFP only was seen (lane 9). However, none of the NS1 RNA-binding domain mutants bound to detectable levels (lanes 13 to 15). Similarly, the effector domain-deleted N81 protein was not apparent in the bound fraction (lane 12), suggesting that (within the limits of detection of the antibody) the truncated protein did not bind NXF1. Thus, there was a

good correlation between the ability of NS1 to promote segment 7 mRNA export and its ability to bind NXF1. This positive correlation supported the hypothesis that NS1 could indeed act as an adaptor protein between segment 7 mRNA and the cellular mRNA export machinery via an interaction with NXF1.

3.2.6 Analysis of the interaction between NXF1 and segment 7 mRNA in the presence of NS1 protein

NXF1 has been shown to bind M1 mRNA in WT virus-infected cells (Wang et al., 2008). Therefore, it was plausible to conjecture that NS1 could facilitate this interaction, acting as an adaptor protein between the viral mRNA and the main cellular mRNA export pathway. To test this, 293T cells were transfected with the plasmids needed to recreate segment 7 RNPs (or with a 2P negative control) along with GFP-NXF1 and, additionally, with or without NS1. Cells were lysed at 48 h p.t. and segment 7 mRNA accumulation was examined by primer extension before or after GFP-Trap affinity purification. Abundant quantities of M1 mRNA and lesser amounts of spliced mRNA2 were present in the total cell lysates from the 3PNP but not the 2P control samples, while examination of cellular 5S rRNA confirmed the extraction of equal cell numbers (**Figure 3.6**, lanes 1 to 3). When the bound fractions were analysed, no M2 mRNA was detected and only trace amounts of M1 mRNA were observed in the absence

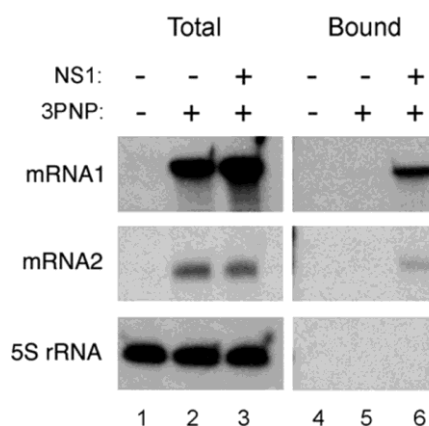


Figure 3.6 Effect of NS1 on the interaction between NXF1 and segment 7 mRNA. 293T cells were transfected with plasmids to recreate segment 7 RNPs (3PNP+) or, as a negative control, with a 2PNP combination lacking a plasmid expressing PB2 (3PNP-) along with GFP-NXF1 and with or without NS1 as indicated. At 48 h p.t., cells were harvested and GFP-Trap pulldown assays were carried out. Subsequently, RNA was extracted and analysed by radioactive reverse transcriptase with primers specific for segment 7 mRNAs and 5S rRNA followed by urea-PAGE and autoradiography.

of NS1. However, upon the presence of NS1, mM1 mRNA was readily detectable (lane 6). M2 could also be detected in the presence of NS1 (lane 6) although to a much lesser extent, suggesting a weaker interaction between NXF1 and M2 mRNA. This suggested that NS1 promoted a stable interaction of NXF1 and segment 7, particularly M1, mRNA.

Having reproduced an NXF1-M1 mRNA interaction and found evidence that NS1 influenced this, the question that naturally followed was whether there was a correlation between the ability of mutant NS1 proteins to promote NXF1-segment 7 mRNA interactions and their mRNA export activity. 293T cells were transfected with either GFP or GFP-NXF1 and 48 h later infected with the panel of WT and NS1 mutant viruses. At 6 h p.i., total RNA was extracted before or after the lysates had been subjected to GFP-Trap pulldown, and primer extension reactions were carried out to assay segment 7 mRNAs and vRNA, as well as 5S rRNA as a loading control. Viral RNAs were detected in the total fraction of every infected sample (**Figure 3.7**, lanes 1 to 7) but only 5S rRNA was detected in mock-infected cells (lane 8). Analysis of the bound fractions from WT virus-infected cells showed that both segment 7 mRNA species co-precipitated with GFP-NXF1 (lane 10). This interaction was specific since

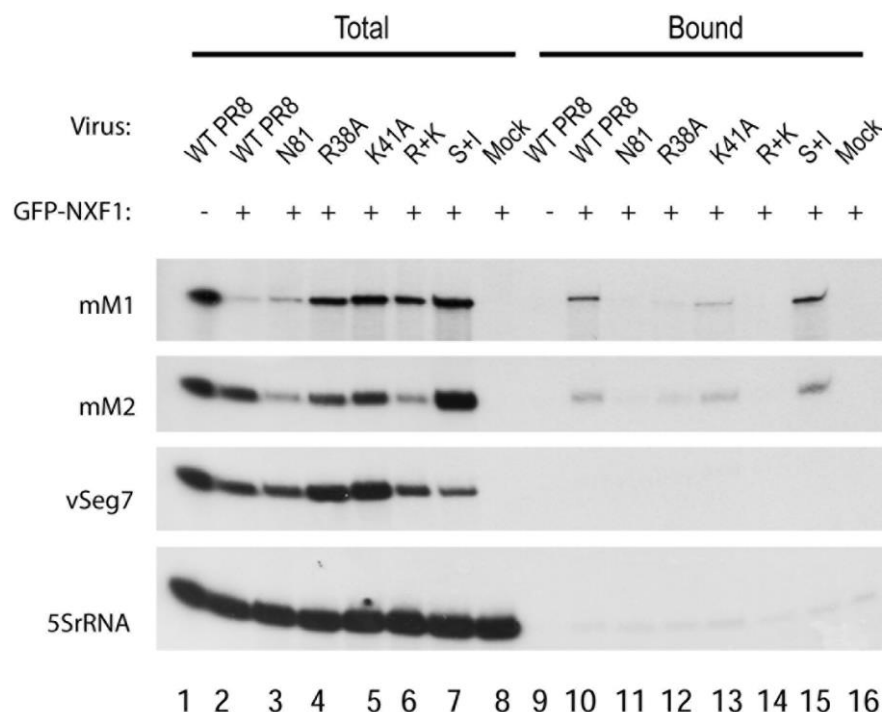


Figure 3.7 Interaction between segment 7 mRNA and NXF1 in the presence of NS1 mutant proteins. 293T cells were transfected with GFP-NXF1 (+) or with GFP alone (-) and 48 h later either mock infected or infected with the indicated viruses at an M.O.I. of 10, in duplicate sets. At 6 h p.i., cells were harvested and total cellular RNA was extracted from one set while the second set was subjected to GFP-Trap pulldown assays prior to total RNA extraction. RNA was analysed by radioactive reverse transcriptase with primers specific for segment 7 mRNAs and vRNA as well as cellular 5S rRNA followed by urea-PAGE and autoradiography.

genomic vRNA did not precipitate with GFP-NXF1 (lane 10) while none of the viral RNA species bound to GFP only (lane 9). A similar outcome was obtained with the export-competent NS1 (S+I) mutant virus (lane 15). However, only trace quantities of M1 or M2 mRNAs bound NXF1 in cells infected with the N81 or R+K NS1 mutant virus (lanes 11 and 14), while reduced amounts co-precipitated from cells infected with the single RNA-binding domain R38 and K41 mutants (lanes 12 and 13). Thus, the ability of NS1 polypeptides to direct efficient export of segment 7 mRNA showed a good correlation with their ability to promote the interaction of the transcripts with NXF1, consistent with the adaptor protein hypothesis.

3.2.7 Ability of NS1 mutant proteins to interact with segment 7 mRNA

Previous work has shown that NS1 binds segment 7 mRNA in vitro and co-precipitates it from infected cells (Burgui et al., 2003; Park and Katze, 1995; Wang et al., 2008). It therefore seemed reasonable to test whether the strength of this interaction correlated with the ability of NS1 mutants to promote efficient export of M1 mRNA. First, an RNP reconstitution assay was used to recreate segment 7 RNPs, with or without the addition of WT or mutant GFP-NS1 polypeptides, before fractionating cell lysates over GFP-Trap beads and analysing the amounts of bound segment 7 mRNA. Examination of aliquots of total cell lysate showed the expected presence of M1 and M2 mRNA species in all samples transfected with all four RNP polypeptides but not in 2P (PB1 and PA) control samples (**Figure 3.8**, lanes 1 to 10). No detectable mRNA co-precipitated with GFP, but easily detectable amounts of both mRNA1 and mRNA2 bound to duplicate samples co-transfected with WT GFP-NS1 or (S+I)-GFP-NS1 (lanes 14, 15, and 20). However, the export-incompetent N81, R38, K41, and R+K mutants all failed to detectably bind M1 mRNA and/or bound greatly reduced amounts of M2 mRNA (lanes 16 to 19). These results suggested that mutant NS1 proteins that failed to promote the efficient nuclear export of segment 7 mRNA were also unable to bind segment 7 mRNA itself, while WT NS1 protein was capable of doing both.

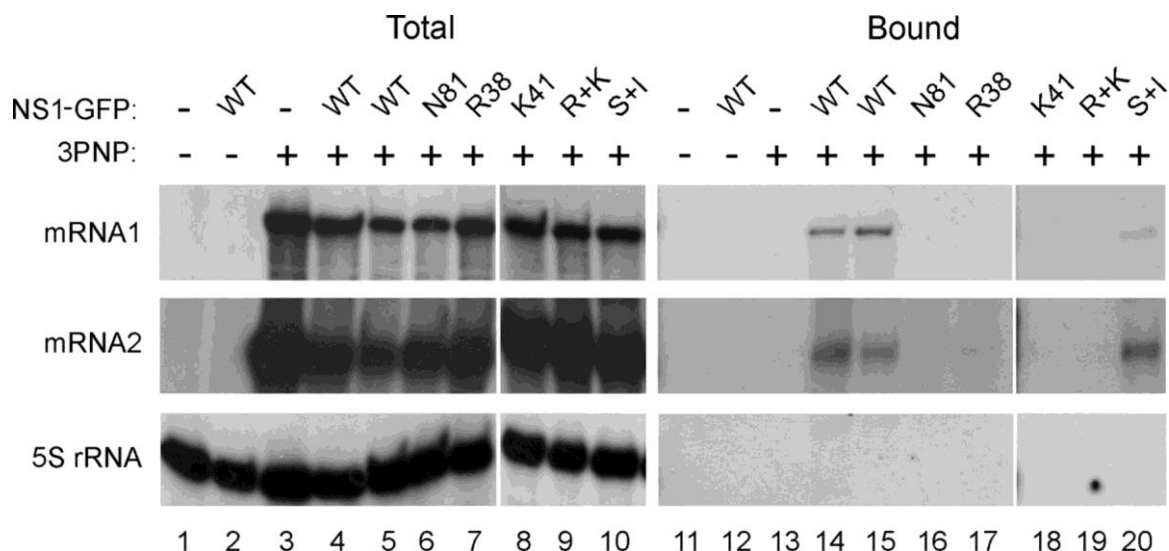


Figure 3.8 Interaction between NS1 and segment 7 mRNA. 293T cells were transfected with plasmids to recreate segment 7 RNPs (3PNP+) or, as a negative control with a 2PNP combination that excluded a plasmid expressing PB2 (3PNP-) together with the indicated NS1-GFP polypeptides (NS1-GFP+) or with GFP (NS1-GFP-), in duplicate sets. At 48 h p.t., cells were harvested and total cellular RNA was extracted from one set while the second set was subjected to GFP-Trap pulldown assays prior to total RNA extraction. RNA was analysed by radioactive reverse transcriptase with primers specific for segment 7 mRNAs and vRNA as well as cellular 5S rRNA followed by urea-PAGE and autoradiography.

3.2.8 Ability of NS1 effector domain mutant proteins to promote efficient export of segment 7 mRNA using the minireplicon system

The evidence so far supported the hypothesis of NS1 acting as an adaptor protein to feed viral segment 7 mRNA(s) to the cellular NXF1 export pathway. To further test the correlation between NS1 export activity and ability to bridge between (or at least bind to both) viral mRNA and NXF1, a set of point mutations in the effector domain of NS1 protein were tested. Mutation of specific amino-acids in the NS1 domain has been shown to result in the loss of several functions attributed to this multifunctional protein (Hale et al., 2008b). The mutants studied here were: NS1-Y89A and NS1-Y89F, both of which prevent NS1 from binding PI3K (Aramini et al., 2011; Hale et al., 2006) NS1-E96/7A preventing NS1 from binding Trim25 (Gack et al., 2009) and NS1-W187R inhibiting the dimerization of NS1 (Hale et al., 2008a).

Firstly, the behaviour of this set of mutant NS1 proteins was analysed in the context of the minireplicon system. 293T cells were transfected as before with plasmids to produce segment 7 RNPs as well as with either WT or mutant NS1-EGFPs. Cells were fixed and processed for FISH at 24 h p.t. in order to observe segment 7 mRNA localisation in the presence of the different NS1 mutant proteins (**Figure 3.9A**). NS1 expression was confirmed by the detection of GFP signal (green) and was found localised predominantly in the

cytoplasm for WT and all mutants except NS1-W187R which presented a mixed phenotype being found both in the nucleus and the cytoplasm. As expected, no mRNA staining (red) was detected in the negative control lacking PB2 (2PNP). When all the basic components for RNP reconstitution were present, segment 7 mRNA could be detected primarily in the cell nucleus, while in the presence of NS1-EGFP, mRNA was found in the cytoplasm. When either of the PI3K-binding mutants NS1-Y89A or NS1-Y89F, were present however, segment 7 mRNA was again found to be clearly retained in the nucleus, indicating that the mutations also resulted in the loss of the ability to promote efficient nuclear export of segment 7 mRNA. Similarly, in the presence

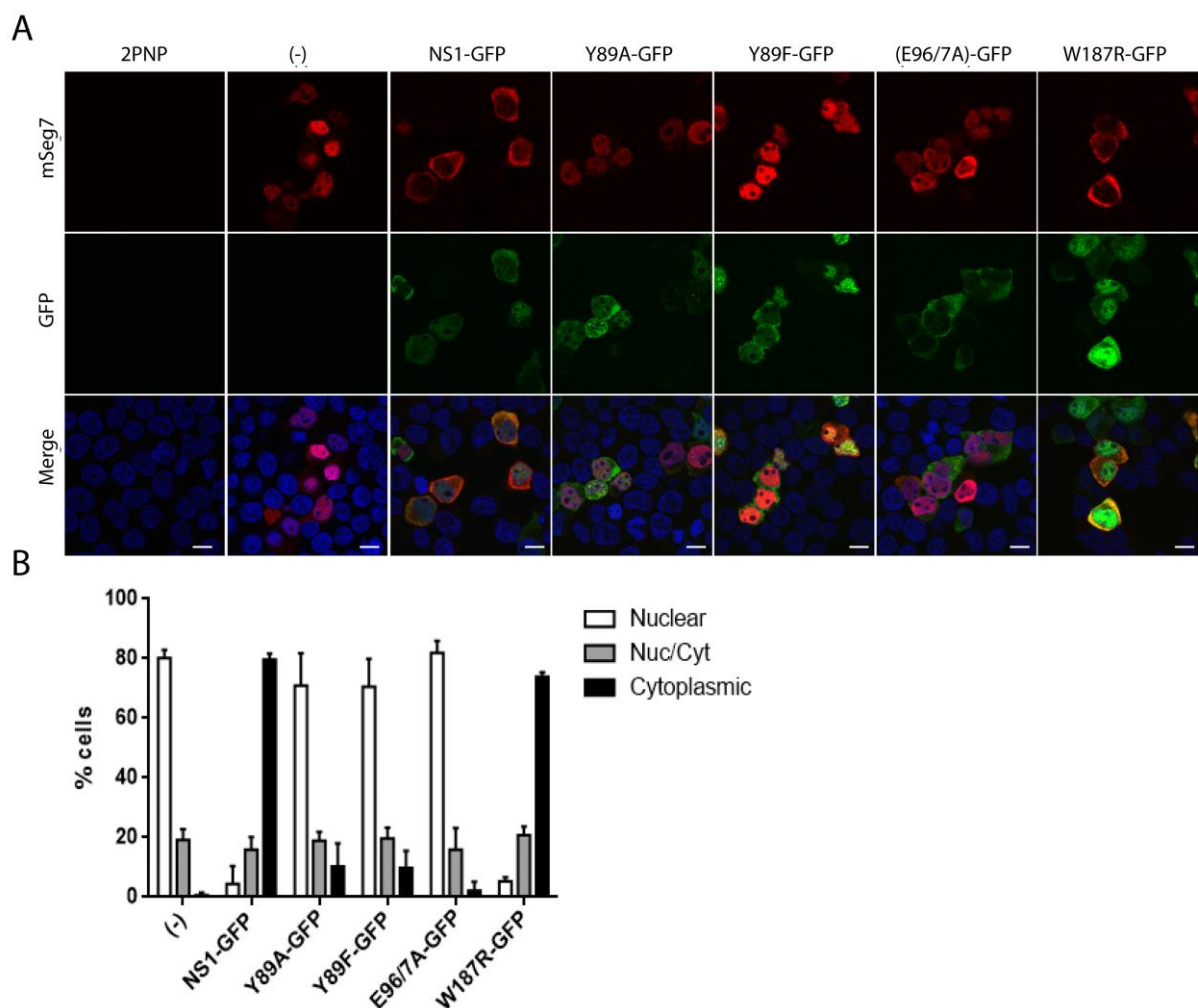


Figure 3.9 Effect of mutations in the NS1 effector domain on segment 7 mRNA localisation. A) 293T cells were co-transfected with plasmids to recreate segment 7 RNPs or, as a negative control with a combination that excluded a plasmid expressing PB2 (2PNP), together with the indicated NS1-GFP polypeptides. Cells were incubated at 37 °C and processed for FISH analysis at 24 h p.t. using Cy3 labelled RNA probes for the detection of positive sense viral RNAs of segment 7 (red) and GFP expression was detected by confocal microscopy (green). Images were captured using a Leica-TCS confocal microscope and Leica TCS analysis software. Scale-bar indicates 10 µm. B) Individual cells were scored according to the predominant cellular localisation of segment 7 mRNA considering three phenotypes: nuclear, cytoplasmic or mixed. Values are the mean ± SEM from two independent experiments.

of the TRIM25-interaction mutant, NS1-E96/7A, the mRNA was also found to be mainly in the nucleus. In contrast, the dimerization mutant NS1-W187R still caused segment 7 mRNA to be localised largely in the cytoplasm, suggesting that the ability of NS1 to dimerise via its effector domain was not required to promote efficient mRNA nuclear export. Cells were counted and scored according to the predominant cellular localisation of segment 7 mRNA (nuclear, cytoplasmic or both), in the presence of the various NS1 mutant proteins (**Figure 3.9B**). These data showed that in the absence of NS1, segment 7 was found in mainly in the nucleus of ~80% of cells scored, while the remaining cells presented mRNA staining both in the nucleus and the cytoplasm. The presence of NS1-E96/7A, NS1-Y89A and NS1-Y89F resulted in a similar outcome with a clear majority of cells displaying segment 7 mRNA in the nucleus. However, more cells transfected with NS1-Y89A and NS1-Y89F displayed cytoplasmic segment 7 mRNA (~10%) than in the complete absence of NS1 or cells transfected with NS1-E96/7A. When WT NS1 or NS1-W187R were present, the clear shift of segment 7 mRNA localisation from the nucleus to the cytoplasm was confirmed. The mRNA was found in the cytoplasm in ~75% of cells and in both cellular compartments in ~20% while less than 10% of cells presented the mRNA solely in the nucleus.

Overall, analysis of this set of mutants confirmed that the effector domain of NS1 is required for the function of mRNA nuclear export, since three NS1 polypeptides with mutations in this domain (NS1-Y89A, NS1-Y89F and NS1-E96/7A) were unable to promote cytoplasmic accumulation of segment 7 mRNA. Furthermore, the results obtained with the NS1-W187R mutant showed that mutations in the effector domain that did not disrupt the nuclear export function were possible, indicating some specificity. Taken together, these data showed that the NS1 effector domain is necessary for the export function and suggested a specific role in viral mRNA nuclear export.

3.2.9 The effect of NS1 effector domain mutant proteins on segment 7 mRNA localisation in the context of viral infection

To further test the mRNA export activity of the NS1 effector domain mutants, their phenotype was examined in the context of virus infection. To this end, 293T cells were mock infected or infected with either WT PR8 or the NS1 mutant viruses NS1-Y89A, NS1-Y89F, NS1-E96/7A and NS1-W187R at an M.O.I. of 5. At 6 h p.i., cells were fixed and processed for FISH analysis to determine the cellular localisation of segment 7 mRNA. In addition, cells were stained by indirect immunofluorescence for NS1 (**Figure 3.10**). All viruses presented

NS1 signal (green) above the uninfected cell background that was generally cytoplasmic, although the staining in cells infected with NS1-Y89 and NS1-E96/7A mutants was noticeably fainter than the WT and W187R viruses. No viral mRNA signal (red) was detected in the mock sample while as expected, in cells infected with WT virus, segment 7 mRNA was

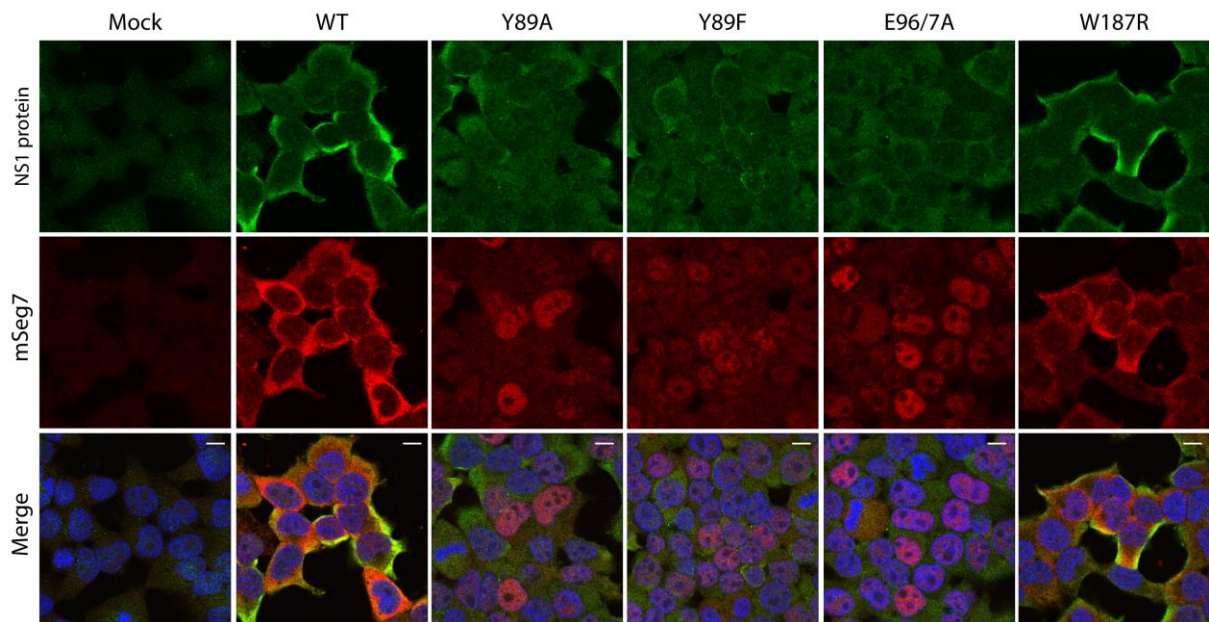


Figure 3.10 Segment 7 mRNA cellular localisation in cells infected with NS1 effector domain mutant viruses. 293T cells were mock infected or infected with the indicated viruses at an M.O.I. of 5. At 6 h p.i., cells were processed for FISH and stained for segment 7 mRNA using a specific RNA fluorescent probe (red) and for DNA using DAPI (blue). Images were captured using a Leica-TCS confocal microscope and Leica TCS analysis software. Scale-bar indicates 10 μ m.

largely found in the cell cytoplasm. In cells infected with the PI3K-binding mutants, NS1-Y89A and NS1-Y89F, segment 7 mRNA was found located predominantly in the cell nucleus. This was also the case in cells infected with the Trim25-interaction mutant NS1-E96/7A, where nuclear retention of segment 7 mRNA was evident. Conversely, analysis of the dimerisation mutant NS1-W187R showed a positive nuclear export phenotype as segment 7 mRNA was mostly found in the cytoplasm, even if some signal was also visible in the cell nucleus. Overall, these results validated the results obtained in the minireplicon setting, as the mRNA export phenotypes of the NS1 mutants were reproduced in the context of viral infection. Furthermore, these data confirmed that not only did NS1 require both functional domains to be able to promote efficient nuclear export of segment 7 mRNA, but also that the introduction of smaller changes in the effector domain could interfere with the export function. The cytoplasmic localisation of segment 7 mRNA in the presence of the NS1-

W187R mutant indicated, however, that NS1 dimerisation was not required in order for NS1 to promote segment 7 mRNA nuclear export.

3.2.10 Analysis of M1 and M2 expression in the presence of effector domain mutant NS1 proteins

Thus far, results indicated a positive correlation between NS1-promoted nuclear export of segment 7 mRNA and higher expression levels of its gene products. In order to determine whether this correlation was maintained with the effector domain mutants, 293T cells were transfected with segment 7 RNPs and the different EGFP-tagged NS1 mutants as before. Western blotting analyses were then performed on cell lysates collected 24 h.p.t.. Equivalent tubulin levels showed that loaded amounts were comparable between all samples (**Figure 3.11A**). The expression of WT and mutant NS1 polypeptides was confirmed by blotting using an antibody against EGFP. Levels of EGFP detected were similar for all the constructs with the exception of NS1-E96/7A, where accumulation was lower (lane 6).

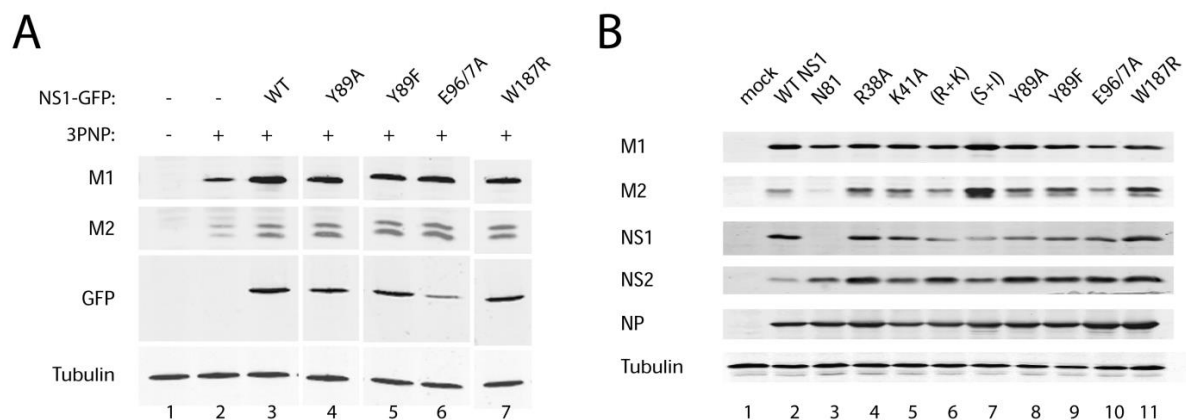


Figure 3.11 Analysis of M1 and M2 expression in the presence of NS1 effector domain mutant proteins. A) 293T cells were co-transfected with plasmids expressing each of the influenza polymerase proteins and NP protein (3PNP) or plasmids expressing PB1, PA and NP proteins (2PNP) along with a plasmid expressing a segment 7 vRNA. Plasmids expressing GFP, NS1-GFP or a GFP-tagged NS1 mutant protein were added as indicated. Cells were incubated at 37 °C, harvested at 24 h p.t. and cell lysates were analysed by western blotting for the proteins indicated. B) 293T cells were either mock infected or infected with NS1 mutant viruses as indicated at an M.O.I. of 5. At 6 h p.i., cells were harvested and cell lysates were analysed by western blotting for the proteins indicated.

Reduced expression of NS1-E96/7A-EGFP was a consistent feature (see later), suggestive of reduced protein stability. A negative control lacking PB2 showed no M gene protein expression as expected (lane 1), but otherwise M1 and M2 expression was readily detected in all samples. As previously, M1 and M2 levels were lower in the absence of NS1 (compare

lanes 2 and 3). When the different NS1 effector domain mutant proteins were present, M2 levels were similar to those seen with WT NS1 (compare lanes 4-7 with lane 3). M1 accumulation was generally intermediate; higher than in the absence of NS1, but lower than in the presence of the WT polypeptide. Notably, there was no obvious difference in M1 accumulation between the mRNA export-competent W187R polypeptide and the inactive Y89 and E96/7 mutants. Thus the correlation between segment 7 mRNA export efficiency and segment protein expression was not maintained when NS1 effector domain mutants were examined in minireplicon reconstitution assays. The next question then was to test whether this correlation also was less evident in the context of viral infection.

To tackle this, 293T cells were mock infected or infected with either WT PR8 or one of the NS1 mutant viruses, at an MOI of 5 and cell lysates harvested at 6 h p.i for western blotting analysis (**Figure 3.11B**). In mock infected cells, no expression of viral proteins was detected, (lane 1) while all viral proteins analysed were present in infected samples (lanes 2-11). Equivalent levels of tubulin were detected in all samples, indicating that the amount of cells collected, lysed and loaded was comparable. While some variability was observed in NP levels across the set of mutants, this variation could not be correlated to the mRNA export phenotype of the mutant viruses and was more likely due to slight discrepancies in the M.O.I. of the viruses. Conversely to the results obtained in minireplicon assays (**Figure 3.11A**), expression of M1 protein showed similar results to previous experiments reported in this dissertation, i.e., lower expression levels were observed in cells infected with viruses carrying an export-incompetent NS1 protein (**Figure 3.11B**, lanes, 3-6 and 8-10). M2 expression levels, however, were lower in cells infected with WT than with most NS1 mutant virus (lane 2). Levels of NS1 protein varied across the different mutant viruses. However, this variation in protein expression levels did not correlate with the ability of NS1 protein to promote efficient export of segment 7 mRNA. For instance, export-incompetent NS1-R38A mutant displayed NS1 levels comparable to WT NS1 whereas export competent NS1-(S+I) showed lower levels of NS1 protein, when compared to WT NS1 (lanes 1, 4 and 7). This indicated that the amount of NS1 protein produced by a virus did not necessarily affect its ability or lack of ability to promote efficient export of segment 7 mRNA. NS2 protein levels were also analysed since segment 8 was mutated to produce the panel of NS1 mutant viruses. All mutant viruses produced higher levels of NS2 compared to WT NS1, so expression levels of NS2 did not appear to be negatively affected by the NS1 mutations or efficient export of segment 7 mRNA being enabled or impaired.

Overall, these results presented the first instance where the results obtained using the minireplicon assay were not in their entirety confirmed by assays of viral infection.

Nevertheless, in the context of viral infection, the correlation between higher levels of M1 protein being produced in the presence of an export-competent NS1 protein was once again observed.

3.2.11 Ability of NS1 effector domain mutant proteins to interact with cellular protein NXF1

Earlier in this chapter, correlative evidence was provided supporting the hypothesis that NS1 acted as an adaptor protein between segment 7 viral mRNA and the cellular mRNA export machinery via its interaction with TAP/NXF1. The variable mRNA export phenotypes of the NS1 effector domain mutants provided a further opportunity to test the correlation between NXF1-binding and export activity. 293T cells were transfected with either GFP or GFP-NXF1 and 48h later mock infected or infected with the panel of NS1 effector domain mutant viruses at an M.O.I. of 5. At 6 h p.i., cells were harvested and lysed and GFP-Trap was performed on the supernatants. Protein analysis was carried out by western blotting which showed that both in the total and bound fractions, GFP and GFP-NXF1 were expressed and collected in comparable amounts as expected. (**Figure 3.12**).

Cells transfected with GFP and infected with WT virus showed the presence of both GFP and NS1 proteins in the total fraction (lane 1). However, only GFP was found in the bound fraction (lanes 1 and 8) thus validating the specificity of the GFP-Trap procedure. In mock infected cells which were used as a further negative control, only NXF1 was detected in both the totals as well as in the bound fractions confirming the absence of infection in these cells (lanes 3 and 10). As expected from previous studies (Larsen et al., 2014; Satterly et al., 2007) and data presented in this dissertation (**Figure 3.5**), WT NS1 co-precipitated with NXF1 (lane 9). Furthermore, all mutant NS1 polypeptides analysed in this experiment also co-precipitated with NXF1 (lanes 11-14). Based on the previous findings reported in this chapter indicating that NS1 mutant proteins that carried the function of viral mRNA export were capable of interacting with NXF1, this outcome was expected for the nuclear export competent dimerization mutant, NS1-W187R. However, the results obtained for the NS1-Y89A, NS1-Y89F and NS1-E96/7A mutant viruses were more interesting since they suggested that although the interaction between NS1 and NXF1 was required for the efficient nuclear export of segment 7 mRNA, it was not sufficient. Therefore other factors and interactions are likely to also play a role in the viral mRNA nuclear export mechanism.

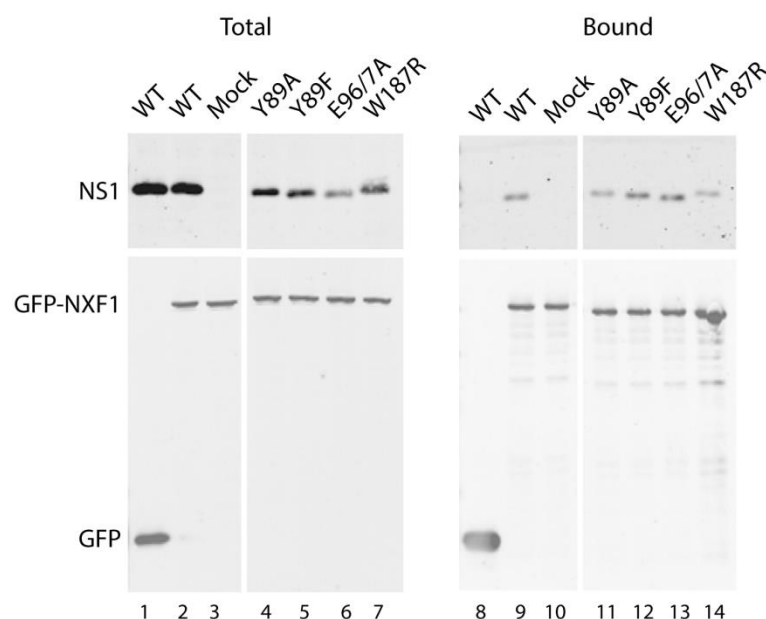


Figure 3.12 Interaction between NXF1 and NS1 effector domain mutant proteins. 293T cells were transfected with plasmids encoding GFP or GFP-NXF1 and 48 h later either mock infected or infected with the indicated NS1 mutant viruses at an M.O.I. of 5. Cells were harvested at 6 h p.i. and cell lysates were examined by western blotting for the indicated proteins before (Total) or after (Bound) being subjected to GFP-Trap pulldown assays.

3.2.12 Effect of NS1 effector domain mutations on the interaction between NXF1 and segment 7 mRNA

The indication from the Y89 and E96/7 mutants that the interaction between NS1 and NXF1 was necessary but not sufficient to enable efficient export of segment 7 meant it was relevant to assess whether they still supported the binding of NXF1 to segment 7 mRNA, to understand if the block in the viral mRNA export pathway was due to the loss of that activity. To test this, 293T cells were transfected with either GFP or GFP-NXF1 and 48 h later mock infected or infected either with WT or NS1 effector domain mutant viruses at an M.O.I. of 10. At 6 h p.i., total RNA was extracted before or after the lysates had been subjected to GFP-Trap pulldown, and primer extension reactions were carried out to examine segment 7RNA species.

Viral RNAs were detected in the total fraction of every infected sample (**Figure 3.13**, lanes 1-2 and 4-7). Analysis of the bound fraction from WT virus infected cells showed that both segment 7 mRNA species co-precipitated with GFP-NXF1 (lane 9) but not with GFP alone (lane 8). The specificity of the interaction between NXF1 and segment 7 mRNAs was further confirmed as genomic vRNA did not co-precipitate with GFP-NXF1 (lane 9) or with GFP alone (lane 8).

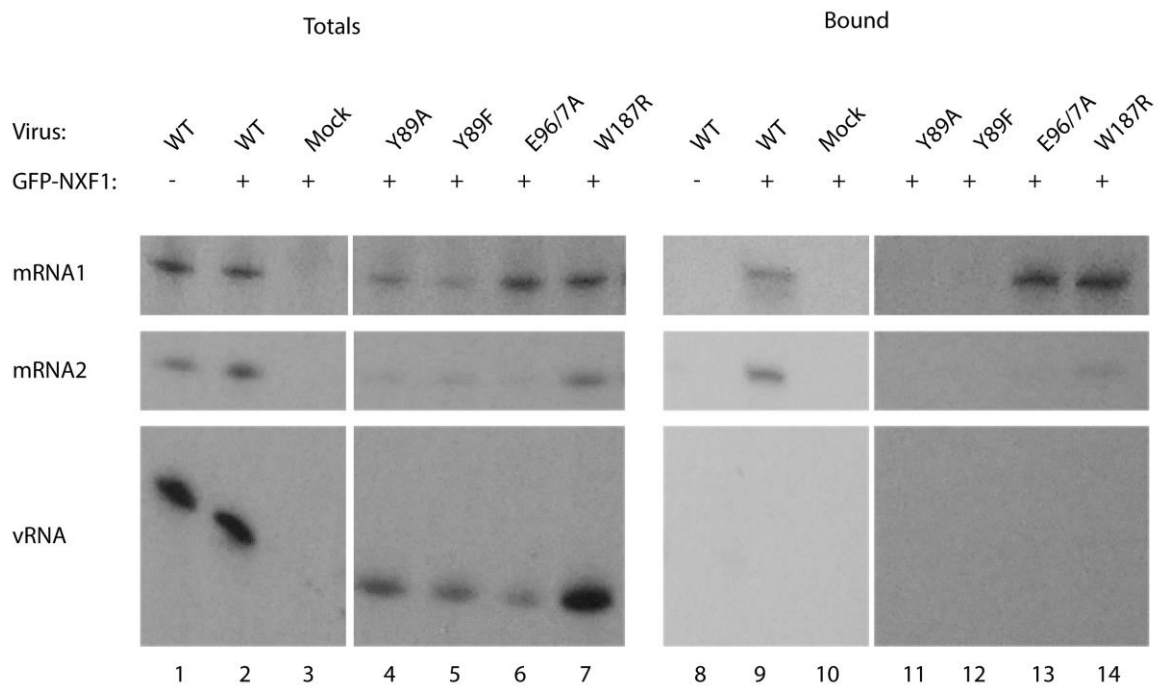


Figure 3.13 Interaction between segment 7 mRNA and NXF1 in the presence of NS1 effector domain mutant proteins. 293T cells were transfected with GFP-NXF1 (GFP-NXF1 +) or with GFP alone (GFP-NXF1 -) and 48 h later either mock infected or infected with the indicated viruses at an M.O.I. of 10, in duplicate sets. At 6 h p.i., cells were harvested and total cellular RNA was extracted from one set while the second set was subjected to GFP-Trap pulldown assays prior to total RNA extraction. RNA was analysed by radioactive reverse transcription followed by urea-PAGE and autoradiography with primers specific for segment 7 RNAs and 5S rRNA.

These results are in line with and further validated the findings described earlier in this chapter (**Figure 3.7**) as well as a previous study (Wang et al., 2008). Analysis of the NS1-W187R mutant showed that NXF1 was able to bind M1 mRNA and, to a lesser extent, M2 mRNA (lane 14). These results were consistent with the ones obtained with the first set of NS1 mutants studied in this dissertation, supporting the hypothesis that NS1 promotes efficient nuclear export of segment 7 mRNA by facilitating the interaction between NXF1 and segment 7 mRNAs. In contrast, viral mRNAs were not detected in the GFP-NXF1 pulldowns from cells infected with either NS1-Y89A or NS1-Y89A mutant viruses (lanes 11 and 12), indicating that both these mutant NS1s failed to promote the interaction between NXF1 and segment 7. This is also in agreement with the findings reported above (**Figure 3.7**), which suggested that NS1 mutant proteins that could not promote nuclear export of segment 7 mRNA were also unable to promote the interaction between NXF1 and segment 7 mRNAs. However, when analysing bound material from cells infected with the NS1-E96/7A virus, M1 mRNA was clearly detected as well as a trace amount of M2 mRNA (lane 13). This latter finding indicated that, like the interaction between NS1 and NXF1, the interaction between NXF1 and segment 7 is necessary, but not sufficient for the successful nuclear export of segment 7 mRNA.

3.2.13 Analysis of NS1 interaction partners

It was thus far observed that various NS1 mutations examined throughout this chapter disrupted the export mechanism for segment 7 mRNA at different steps. The RNA-binding domain mutations seemed to halt the nuclear export of segment 7 mRNA by making NS1 unable to bind NXF1 and/or segment 7 mRNA. The NS1 effector domain mutants however, were all able to interact with NXF1. In this instance, the lack of interaction between NXF1 and segment 7 mRNA in the presence of the mutants NS1-Y89A and NS1-Y89F could be what interrupted viral mRNA nuclear export. In the case of the NS1-E96/7A mutant virus however, it remained unclear what caused its loss of viral mRNA nuclear export function. The mutant NS1 protein was able to bind NXF1 and also to promote the interaction between NXF1 and segment 7 mRNA, suggesting the hypothesis that an as yet unidentified interaction with a further factor necessary for viral mRNA export was affected by the E96/7A mutation. Examination of the cellular interaction partners of the various WT and mutant NS1 polypeptides might therefore be expected to show differences, in particular the E96/7A protein. Accordingly, 293T cells were transfected either with GFP or GFP-tagged NS1 mutant plasmids. At 48 h p.t., cells were collected, lysed and subjected to GFP-Trap. The bound fractions were loaded onto SDS-PAGE gels and stained with Coomassie Blue dye. GFP was the only protein detected from cells transfected with the GFP control plasmid, indicating no obvious contamination with cellular proteins that bound specifically to GFP (**Figure 3.14**, lane 1). Major polypeptide species of the expected size for GFP-NS1 were detected in all other samples, including the truncated form NS1-N81, confirming that cells were successfully transfected (lanes 2-9). Various other minor polypeptide species were also visible that were mostly common to all NS1-containing samples. Examination of this and replicate experiments (data not shown) generally found only minor differences in the polypeptide profiles that could not be correlated with the viral mRNA nuclear export activity of the particular NS1. However, one clear difference was consistently observed; a distinct novel polypeptide was detected in pulldowns from cells transfected with E96/7A (lane 8) that was absent from the other samples. Since NS1-E96/7A was precisely the mutant that posed most questions, being unable to promote nuclear export of segment 7 while being able to bind NXF1 and promote the interaction between NXF1 and segment 7 mRNA, the identification of this protein was pursued.

Thus, a further aliquot of the NS1-E96/7A sample was run in another gel and sent to the Roslin Institute Proteomics Facility which unambiguously identified the mystery polypeptide as heat shock protein 70 (hsp70) through multiple peptide matches after liquid chromatography mass spectrometry analysis of the excised band (data not shown).

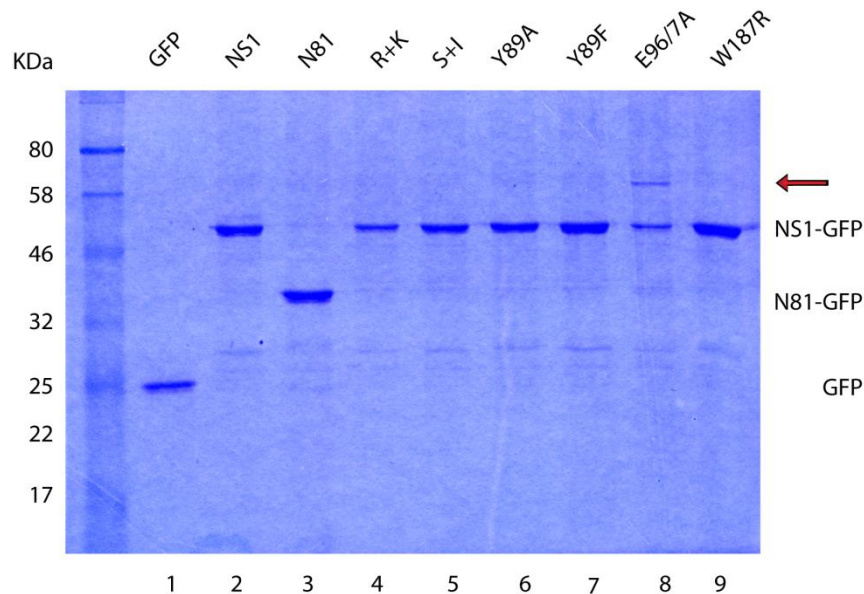


Figure 3.14 Analysis of NS1-GFP-interacting polypeptides. 293T cells were transfected with either GFP alone or with GFP-tagged NS1 mutant protein expressing plasmids as indicated and 48 h later cells were harvested and subjected to GFP-Trap pull down assays. Eluted samples were loaded onto SDS-PAGE gels and stained with Coomassie Blue dye. The red arrow indicates the unique polypeptide species that co-purified with NS1-E96/A (lane 8) that was sent to the Roslin Institute proteomics facility which identified it as hsp70.

To confirm the interaction between NS1-E96/7A and hsp70, the GFP-trap pulldowns were repeated and analysed by western blotting for hsp70 as well as for the GFP-tagged “bait” proteins. In addition, because analysis of the overall protein-binding profiles of the various mutant NS1 proteins had not revealed any other striking differences that could unveil a crucial interaction for the viral mRNA export pathway, specific cellular proteins for which working antisera were available were also examined. Accordingly, 293T cells were transfected either with GFP or GFP-tagged NS1 mutant plasmids. At 48 h p.t., cells were collected, lysed and subjected to GFP-Trap followed by western blotting. Transfection was confirmed by the detection of GFP and GFP-tagged NS1 mutant proteins, while equivalent amounts of the cellular proteins hsp70, Nup62 and UAP56 in the total fractions of all samples confirmed that comparable amounts of cell lysates had been generated (**Figure 3.15**, lanes 1-9). However, in the bound fraction, hsp70 was detected only in the NS1-E96/7A sample (lane 17). These results confirmed that the interaction between NS1-E96/A and hsp70 existed and that it was specific to this particular NS1 mutant protein. In contrast, Nup62 was detected, albeit in trace amounts, in all samples apart from the one from cells transfected with GFP (lanes 10-18), suggesting a weak but NS1-specific interaction. UAP56 on the other hand, could not be detected in any of the bound fraction samples, suggesting that NS1 protein does not interact with it. Taken together, these results confirmed the specific gain-of-function interaction with

hsp70 for NS1 E96/7 and also suggested that NS1 was able to interact with a cellular component heavily implicated in the cellular mRNA export pathway Nup62 (Satterly et al., 2007). However, since all NS1 mutants were able to support this latter interaction, no mechanistic connection with viral mRNA export could be made.

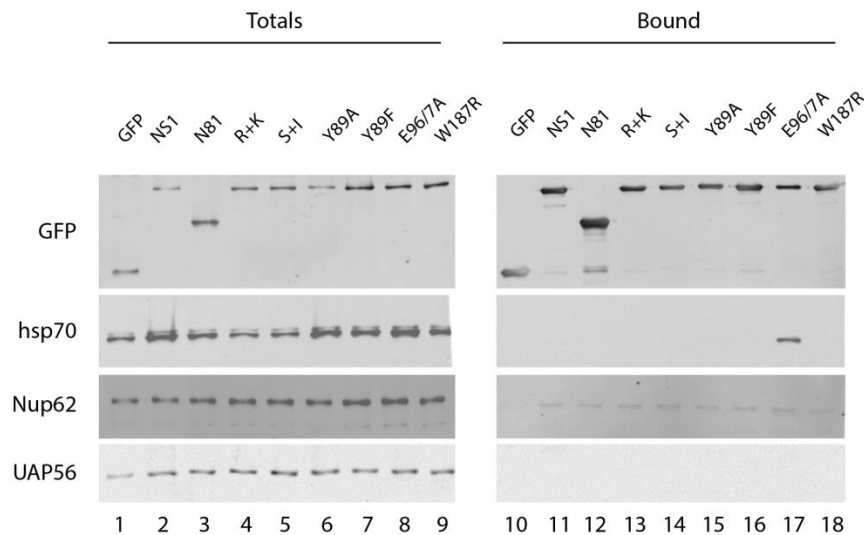


Figure 3.15 Analysis of NS1 interactions with cellular factors. 293T cells were transfected with GFP or with GFP-tagged NS1 mutant proteins expressing plasmids as indicated and 48 h p.t., cells were harvested and cell lysates examined by western blotting for the indicated proteins before (Total) or after (Bound) being subjected to GFP-Trap pulldown assays.

3.3 Discussion

The aim of the work presented in this chapter was to gather a deeper understanding of the mechanism by which segment 7 mRNA is exported from the nucleus to the cytoplasm following the identification of NS1 as a viral requirement for this process in Chapter 2. Since a panel of NS1 mutants (in the form of plasmid constructs as well as viruses) were already available in the Digard laboratory, these were used to tackle NS1 protein itself. The objective was to study the impact of mutations thought to inhibit specific roles attributed to NS1 and assess whether these mutations also affected the ability of NS1 to promote efficient nuclear export of segment 7 mRNA.

Initially, the focus was on major mutations that would separately test the role of the two functional domains of NS1 (Qian et al., 1994; Qian et al., 1995) in order to identify which of them was associated with the mRNA nuclear export function. Positively-charged residues at positions 38 and 41 have been identified as required for the integrity of the RNA-binding domain and substitution of both the arginine at position 38 and the lysine at position 41 for

alanine was shown to disrupt the RNA-binding ability of NS1 (Hale et al., 2008b; Wang et al., 1999). Hence, three NS1 mutants were used to test the impact of losing a functional RNA-binding domain in the context of mRNA nuclear export: NS1-R38A, NS1-K41A and the double mutant NS1-(R+K). Abrogation of the entire effector domain required a more drastic change to be introduced onto NS1. A stop codon was introduced at position 82 resulting in a truncated NS1 protein comprising the first 81 amino acids of the full-length polypeptide (Zurcher et al., 2000). This mutant protein, NS1-N81, contained an intact RNA-binding domain but lacked the entire effector domain. Studying the cellular localisation of segment mRNA by FISH in the presence of these NS1 mutant proteins revealed that neither of them was able to support the export of the mRNA to the cytoplasm, resulting in a marked nuclear retention of the mRNA (**Figure 3.1** and **3.3**). This suggests that both the RNA-binding and the effector domains of NS1 are required for the function of viral mRNA nuclear export. Similarly, the splicing inhibition function attributed to NS1 has also been reported to require both functional domains of the protein (Lu et al., 1994).

NS1 has previously been hypothesized to play a role in IAV mRNA nuclear export, although this had not been clearly demonstrated prior to the work described in this Chapter (Schneider and Wolff, 2009). The identification of interactions between NS1 and viral mRNAs (Burgui et al., 2003; Marion et al., 1997; Park and Katze, 1995; Wang et al., 2008) as well as between NS1 and NXF1 (Larsen et al., 2014; Satterly et al., 2007; Wang et al., 2008) provided strong indications that NS1 could facilitate the nuclear export of viral mRNAs via its interaction with NXF1 and other components of the cellular mRNA export machinery. Here, these interactions were corroborated and it was confirmed that WT NS1 interacts both with NXF1 and segment 7 mRNA in the experimental conditions described (**Figures 3.5** and **3.8**). Functional understanding of these interactions was further extended by showing that not only can NS1 bind segment 7 mRNA and NXF1, but that NS1 also promotes the interaction between NXF1 and segment 7 mRNA (**Figures 3.6** and **3.7**). As both functional domains of NS1 were demonstrated to be required for efficient segment 7 mRNA nuclear export to occur, it was plausible to consider that at least one but perhaps both would be also necessary for the interaction between NS1 and NXF1. Indeed, neither of the RNA-binding mutants nor the effector domain mutant could detectably interact with NXF1 (**Figure 3.5**) indicating that both functional domains are necessary for stable complex formation. Similarly, the three RNA-binding mutants as well as the effector domain mutant were unable to interact with segment 7 mRNA (**Figure 3.8**) and also failed to promote the interaction between segment 7 mRNA and NXF1 (**Figures 3.7** and **3.8**). These results suggested that, in order for segment 7 mRNA to be

efficiently exported to the nucleus via NS1, it was necessary to comply with at least three conditions: 1) NS1 must be able to interact with NXF1, 2) NS1 must be able to bind segment 7 mRNA and 3) NS1 must be able to promote the interaction between segment 7 mRNA and NXF1. Failing to present any of these three features would result in segment 7 mRNA nuclear retention. Moreover, results suggest that both functional domains of NS1 must be active in order to fulfil the criteria. However, in a previous report, the interaction between NS1 and NXF1 was found to rely solely on the RNA-binding domain of NS1 (Satterly et al., 2007). Since the effector domain mutant used, NS1-N81, produced a truncated form of NS1 protein that was not easily detectable by western blotting with the antisera available in the laboratory, it is possible that an interaction between NXF1 and the first 81 amino acids of NS1 existed that could not be observed in the assays performed here. Nevertheless, even if NS1-N81 could bind NXF1, the latter failed to interact with segment 7 mRNA in the presence of NS1-N81. Therefore, the effector domain of NS1 does play a role in these interactions; if not directly in the interaction between NS1 and NXF1 or NS1 and segment 7 mRNA, then at least for the interaction between NXF1 and segment 7 mRNA. These findings were in favour of a model by which NS1 interacts with segment 7 mRNA and transfers it to NXF1 as a strategy to feed the viral transcript into the major cellular mRNA export pathway. It is however important to note that these interactions were not tested *in vitro* and thus, it was not possible to establish that they were direct. It is possible that these interactions could be mediated by yet another cellular (or viral, if present in the minimal IAV RNP set of genes) component.

To date, the RNA-binding domain of NS1 has been shown to interact with multiple RNA species such as double-stranded RNAs (Chien et al., 1997; Hatada and Fukuda, 1992; Wang et al., 1999), small nuclear RNAs (Qiu et al., 1995; Wang and Krug, 1998), viral genomic RNAs (Hatada et al., 1997; Hatada et al., 1992) and viral mRNAs (Burgui et al., 2003; Marion et al., 1997; Wang et al., 2008). However, the specific residues of NS1 involved in these interactions have not yet been identified. While it was previously thought that the RNA-binding domain of NS1 interacted with RNAs in a sequence independent fashion, a more recent study suggested that the interactions were specific, showed a very high affinity and were structure-dependent (Marc et al., 2013). Hence it is plausible to conjecture that NS1 could bind segment 7 mRNA via its RNA-binding domain through the recognition of a specific sequence or structure within the mRNA, most likely in its 5'-UTR (Marc et al., 2013; Park and Katze, 1995). Another possibility is that NS1 binds segment 7 mRNA via its poly(A) tail. An interaction between NS1 and poly(A) sequences of cellular and viral mRNAs has been demonstrated and postulated to result in the inhibition of nuclear export of all

mRNAs containing a poly(A) tail, including viral mRNAs (Qiu and Krug, 1994). However, the results obtained in Chapter 2 point in a different direction, as the presence of NS1 could not rescue the nuclear export of segment 7 mRNA when it contained a poly(U) tail. This indicated that a poly(A) tail is in fact required for NS1 to be able to promote the nuclear export of segment 7 mRNA (**Figure 2.7**), either because NS1 binds to this mRNA sequence directly to promote rather than inhibit viral mRNA export or perhaps because NS1 needs to interact with a cellular factor that associates with a poly(A) tail. NS1 protein was later proposed to inhibit cellular, but not viral, mRNA formation by targeting CPSF30, a subunit of CPSF. Binding of NS1 to this subunit was shown to prevent CPSF from interacting with host pre-mRNAs, thereby inhibiting their 3' cleavage and subsequent polyadenylation (Nemeroff et al., 1998). NS1 has also been shown to interfere with the cellular 3' end processing machinery via targeting the poly (A) binding protein II (PABPII) resulting in the inhibition of the elongation of poly(A) tails catalysed by poly(A) polymerase (PAP) (Chen et al., 1999). Therefore although a positive function of an NS1-poly(A) tail during viral mRNA export cannot be ruled out, further work should probably prioritise the functional significance of the published interactions between NS1 and the 5'UTR of segment 7 mRNAs (Marc et al., 2013; Park and Katze, 1995) for nuclear export.

The IAV strain used here was PR8, a strain that expresses an NS1 that lacks the ability to bind CPFS30 (Kochs et al., 2007). When selecting the panel of NS1 mutations to be examined in the context of segment 7 mRNA export, an NS1 mutant that would allow testing for an interplay between effects on cellular polyadenylation and viral mRNA export was included. This NS1 protein had two substitutions: phenylalanine was replaced by serine at position 103 and methionine was substituted by isoleucine at position 106 resulting in the NS1-(S+I) mutant protein. These mutations were previously demonstrated to restore the ability of PR8 NS1 to bind CPSF30 and thereby inhibit cellular mRNA polyadenylation (Kochs et al., 2007). Work carried out by other members of the Digard laboratory showed that these mutations did indeed confer the expected shut-off activity on the NS1 clone used here (personal communication, Dr Liliane Chung). The NS1-(S+I) mutant gave positive results for all tested activities regarding mRNA nuclear export. Segment 7 mRNA was found in the cytoplasm in the presence of NS1-(S+I) and the latter was able to interact with NXF1, segment 7 mRNA and to promote the interaction between NXF1 and segment 7 mRNA (**Figures 3.1, 3.3, 3.5, 3.7 and 3.8**). However, the restoration of NS1 binding ability to CPSF30 did seem to have an effect on NS1-mediated segment 7 mRNA cellular localisation; it appeared to actually improve the efficiency of mRNA nuclear export. Although segment 7

mRNA was clearly found in predominantly in the cytoplasm upon the presence of WT NS1 and NS1-(S+I), less nuclear staining was found in cells either transfected or infected with NS1-(S+I) (**Figures 3.1 and 3.3**). Furthermore, the interaction between NS1-(S+I) and NXF1, as well as the interaction between NXF1 and segment 7 mRNA, appeared to be somewhat enhanced by NS1-(S+I) when compared to WT NS1 (**Figures 3.5 and 3.7**). However, the reasons behind this were not explored as the focus was more directed at understanding what caused NS1 mutants to fail to promote nuclear export of segment 7 mRNA rather than enhance it. In addition, the proposed phenotype of improved activity of the NS1-(S+I) mutant remained qualitative, as attempts to provide more quantitative data for the RNA pulldown assays (by densitometry of the various autoradiograms) were defeated by the technical difficulty of the assays and the consequent inherent variability of the data. Nonetheless, it is conceivable that NS1 binding CPSF30 favours the nuclear export of viral mRNAs by increasing the availability of components of the cellular mRNA export machinery. Since the interaction between NS1 and CPSF30 interferes with the 3' end processing of cellular mRNAs, resulting in the inhibition of their export (Nemeroff et al., 1998), viral mRNAs might have less competition for cellular factors involved in mRNA nuclear export.

Given that the initial take on the requirement of the effector domain of NS1 was based on the introduction of a mutation that deleted the entire domain, a second set of NS1 mutants was used to study this function of the domain in connection with mRNA nuclear export in greater detail. Firstly, a preliminary panel of NS1 proteins carrying mutations in the effector domain that was already available in the Digard laboratory was screened. Based on the expression levels of these NS1 mutant proteins and their effect on segment 7 mRNA localisation, four NS1 mutants with the most stable protein expression and clearest export phenotype were selected for further examination. Two of the mutations inhibited the ability of NS1 to activate the PI3K pathway by blocking its interaction with the p85 β regulatory subunit of PI3K (Shin et al., 2007): NS1-Y89A (Aramini et al., 2011; Lopes et al., 2017) and NS1-Y89F (Hale et al., 2006). The third, NS1-E96/7A, blocked the ability of NS1 to suppress TRIM25-mediated RIG-I signal transduction, resulting in the inhibition of host IFN response and attenuated virulence in mice (Gack et al., 2009). Finally, the fourth mutation, NS1-W187R prevented NS1 dimerisation via its effector domain (Hale et al., 2008a). Cells containing an NS1-W187R mutant displayed segment 7 mRNA in the cytoplasm (**Figures 3.9 and 3.10**), indicating that NS1 dimerisation via its effector domain is not required for this protein to mediate efficient mRNA nuclear export, although it is worth noting that NS1 dimerization via the RNA-binding domain has also been reported (Kerry et al., 2011a).

Conversely, in the presence of both PI3K NS1 as well as the TRIM25 mutants, segment 7 mRNA was found to be retained in the cell nucleus (**Figures 3.9 and 3.10**). These results could indicate that the activation of the PI3K signalling pathway and/or the inhibition of the interaction between NS1 and TRIM25 are somehow involved in facilitating viral mRNA nuclear export. An attempt was made to determine the effect of small-molecule inhibition of PI3K signalling on segment 7 mRNA localisation in cells infected with WT PR8. Unfortunately, results were inconsistent (data not shown) which prevented any conclusions being drawn. Alternatively, the mutated residues could in parallel disrupt a potential binding site for another cellular factor participating in the viral mRNA nuclear export pathway. This could be either because the residues are critical for this other interaction or perhaps due to conformational changes that would abolish the binding site of this other NS1 binding partner.

Thus, the question of whether this second panel of NS1 mutants were able to support the interactions or lack thereof that had been previously established for the RNA-binding domain mutants and NS1-N81 was investigated. It was found that NS1-W187R behaved as expected for a protein capable of supporting segment 7 mRNA nuclear export. As with WT NS1, NS1-W187R was able to interact with NXF1 and to promote the interaction between NXF1 and segment 7 mRNA (**Figures 3.12 and 3.13**). More surprising however, was the observation that both the PI3K and the TRIM25 NS1 mutant proteins were able to interact with NXF1 (**Figure 3.12**) even though they failed to promote the efficient nuclear export of segment 7 mRNA. Curiously, export-incompetent TRIM25 mutant NS1-E96/7A, similarly to WT NS1 and NS1-W187R, was capable of promoting the interaction between NXF1 and segment 7 mRNA. Determination of whether these NS1 effector domain mutant proteins were capable of interacting with segment 7 mRNA was also attempted. However, results from those experiments showed substantial variability in their outcome and provided no confidence in determining the presence or absence of interactions (data not shown). Hence, these data were not included in this dissertation.

The results that were obtained reproducibly, however, were unexpected but very interesting. On the one hand, they further confirmed and established that both functional domains of the NS1 protein are required for it to retain its mRNA export function. While it was not possible to pinpoint how many residues or regions needed to be kept intact within the effector domain, it was shown that at least substitutions at positions 89 and 96-97 resulted in segment 7 mRNA nuclear retention. It is worth noting that it was not possible to discern whether the mRNA nuclear retention observed in the presence of the mutant proteins was

residue substitution-specific or in any way connected with the other NS1 functions relying on the same residues. To date (to the best of my knowledge), no direct link has been demonstrated between either the activation of the PI3K signalling pathway activation or TRIM25 inhibition and IAV mRNA nuclear export.

On the other hand, these results demonstrate that while the interactions between NS1 and NXF1 and NXF1 and segment 7 mRNA are required for the efficient export of segment 7 mRNA, they are not sufficient. Indeed, it is possible that a mutant NS1 protein can bind NXF1 and promote its interaction with segment 7 mRNA while still failing to perform its role in facilitating mRNA nuclear export. The realization that one or more other interactions would be necessary to complete the mRNA nuclear export mechanism as a whole was not surprising as the involvement and requirement of other cellular factors has been demonstrated or suggested before (Amorim et al., 2007; Bier et al., 2011; Mor et al., 2016; Read and Digard, 2010; Satterly et al., 2007; Wang et al., 2008). However, data presented in this chapter suggests that the role of NS1 itself in this mechanism is not limited to its interaction with NXF1 as perhaps indicated by the analysis of the first set of NS1 mutants. It is instead probable that NS1 mediates a further interaction or recruits an additional cellular component possibly, but not necessarily, downstream of its interaction with NXF1 in order to fulfil its function of promoting segment 7 RNA nuclear export.

The findings presented in this chapter show that, in order to be export-competent, NS1 is required to interact with NXF1, with segment 7 mRNA and to promote the interaction between NXF1 and segment 7 mRNA. However, these interactions were not sufficient and an attempt was made to identify another cellular factor that would be required to interact with NS1 and that could be correlated to its ability to promote mRNA nuclear export. When examining the protein profiles of cellular polypeptides that co-purified with one of the entire panel of NS1 mutants during GFP-trap pulldowns, it was found that they appeared to be generally the same and not much variation was seen in band intensity or presence/absence of minor polypeptide species when comparing all the mutants (**Figure 3.14**). However, the one notable, observable to the naked eye upon visualization of the Coomassie stained gel, difference was the appearance of one extra band in cells transfected with the TRIM25 NS1 mutant, that was absent from all the other samples. Mass spectrometry analysis revealed that this band corresponded to hsp70. This could indicate that this protein is misfolded which could explain its lower expression levels (**Figure 3.11A**). One report has shown that hsp70 induction is suppressed in cells infected with IAV, by inhibiting the cleavage of hsp70 pre-

mRNAs at the polyadenylation site (Shimizu et al., 1999). Results from this study supported the argument that viral infection induced the shut off of host gene expression by interfering with the 3' end processing of pre-mRNA, but no connection was made between hsp70 suppression and viral mRNA export. However, in this same study a temperature sensitive Udorn NS1 mutant was found to not inhibit the hsp70 pre-mRNA cleavage at 40 °C in MDCK cells. Interestingly, when the mutations responsible were examined, two base changes were identified, one at base 62 that did not cause a residue change and another at base 316 that caused an amino acid change at residue 96, one of the residues mutated in the PR8 NS1-E96/7A that was found to interact with hsp70 in 293T cells. It is not clear if this is functionally relevant to the studies presented here, but the coincidence is intriguing.

The interaction observed between NS1-E96/7A and hsp70 could merely indicate that the protein is not properly folded, as hsp70 is associated with assisting with multiple protein folding processes (Mayer and Bukau, 2005). Recently, a report has shown that hsp70 can suppress protein aggregation by protecting partially folded and unfolded protein chains, affect late stages of protein folding and that it can both stabilise and destabilise folded structures in a nucleotide-dependent manner (Mashaghi et al., 2016). It is thus not clear what the implications of NS1 interaction with hsp70 could be and discerning if this interaction is significant would need more detailed studies in the future. Nevertheless, this interaction could be meaningful as it was present with the single mutant NS1 protein studied in this dissertation that could interact with NXF1 and promote the interaction between NXF1 and segment 7 mRNA yet failed to promote the efficient export of segment 7 mRNA. It can be speculated that the interaction between NS1 and hsp70 results in a change in the conformation of NS1 that while still allowing for its interaction with NXF1, perhaps interferes with its ability to interact with another factor necessary to complete the mRNA export mechanism. Alternatively, residues 96 and 97 of NS1 could be part of the binding site for another interacting partner playing a role in segment 7 mRNA export and mutation of these residues would result in hsp70 outcompeting this partner for the binding site. It would be interesting to test the effects of the growing numbers of small molecule inhibitors of hsp70 now available (Chatterjee and Burns, 2017) on the behaviour of the NS1-E96/7A mutant, though the likelihood of pleiotropic effects might be high.

The analysis of the overall expression profiles of cells presenting the various NS1 mutants did not point towards major differences that could be correlated to the ability or lack thereof of NS1 promoting the efficient nuclear export of segment 7 mRNA. Hence, an attempt

was made to analyse the ability of NS1 to interact with the most obvious suspects involved in cellular mRNA export. During mRNA synthesis and processing, IAV transcripts interact with multiple cellular proteins (York and Fodor, 2013) and many of them could play a role in the NS1-mediated viral mRNA export. Cellular proteins NXF1, Nxt1, Rae1 and E1B-AP5 have been reported to interact with NS1 (Satterly et al., 2007). Together with Nup98, these cellular factors were previously shown to be able to form a complex (Blevins et al., 2003). The same study proposed that Rae1 could deliver NXF1 to Nup98 representing the first of several interactions between an export complex and a nucleoporin that would ultimately result in the nuclear export of cellular mRNAs. Furthermore, reduction of UAP56 and NXF1 were shown to inhibit the mRNA nuclear export of several IAV segments (Read and Digard, 2010). Results indicated that neither of the NS1 mutant proteins studied in this Chapter were able to interact with UAP56 (**Figure 3.15**), even though the lack of this factor was shown to strongly inhibit nuclear export of both spliced and unspliced segment 7 mRNAs (Read and Digard, 2010). Alternatively, the known interaction between NP and UAP56 (Momose et al., 2001) may be functionally relevant here. Furthermore, Nup 62 was detected, albeit in low amounts in cells transfected with all NS1-mutants, but not with GFP, indicating that NS1 can bind this nucleoporin, even if weakly (**Figure 3.15**). However, since all NS1 mutants could support this interaction, it does not seem to be correlated with the ability of NS1 to promote efficient nuclear export of segment 7 mRNA.

Despite the long list of candidate proteins, the spectrum of the analysis was very limited. One of the main reasons for this was that the work was restricted by available reagents. For instance, several attempts were made to study a potential interaction between NS1 and Nxt1, the co-factor of NXF1 (Herold et al., 2001; Suyama et al., 2000), both in IF as well as in western blotting. However, despite testing many different commercial antibodies, it was not possible to obtain specific detection of this cellular factor in multiple assays. Similar difficulties were encountered when attempts to test the interaction between NS1 and UIF, PABP1, Aly and Nup98. It was not possible to gather conclusive information as whether these proteins could interact with NS1 as antisera against them either failed to show specificity or failed to work reproducibly. It is also worth noting that a potential shortcoming of this assay and the overall expression profile of cells transfected with NS1 mutant proteins was that it was not done in the context of viral infection. Samples lacked all IAV components except NS1 and it is therefore possible, or perhaps even probable, that, potential differences in the expression profiles or interactions were missed. For instance, specific interactions of NS1 associated with its role of promoting efficient segment 7 mRNA export may only be evident

in infected (or at least RNP-containing) cells, due to viral components being present that would trigger specific pathways/mechanism. One way to get around this would be to infect cells with the NS1-N81 mutant virus following the transfection of cells with the GFP-tagged NS1 mutant proteins. However, this mutant still produces a truncated form of NS1 containing the RNA-binding domain which could possibly maintain interactions and mask the phenotype of the other mutations under study. Alternatively, the minireplicon assay could be used although this assay would also not mimic a viral infection and again, important interactions that occur during infection could be missed. Hence, the list of possibilities for protein associated to the cellular mRNA export machinery that could intervene in IAV mRNA export via NS1 continues to be extensive. It would be interesting for studies to continue in order to identify NS1 partners in the mRNA nuclear export mechanism. Since it was shown in this Chapter that the participation of NXF1 in connection with NS1 is required but not sufficient, it can be speculated that a further unknown cellular (or even viral) component would act downstream of NS1 interacting with NXF1.

Both in Chapter 2 and in this Chapter, NS1 was established as a viral requirement for the efficient nuclear export of segment 7 mRNA to occur. However, it was observed in both Chapters that, upon infection with NS1-N81, even though segment 7 mRNA was found markedly retained in the nucleus, significant levels of segment 7 gene products, M1 and M2 were expressed (**Figures 2.9, 3.2 and 3.4**). Moreover, results described in this Chapter, showed that similarly, all NS1 point mutants that resulted in a clear nuclear retention of segment 7 mRNA were also able to produce both M1 and M2 proteins, although at lower levels when compared to export-competent NS1 mutants (**Figures 3.2, 3.4, 3.11**). This indicates that some mRNA is able to be exported to the cytoplasm for translation via a mechanism that does not rely on NS1. The shift in cellular localisation of segment 7 mRNA in the absence of NS1 when compared when WT NS1 protein is present is evident, suggesting that the mechanism involving NS1 in the mRNA nuclear export is more efficient and is favoured in a viral infection. Nonetheless, a different, NS1-independent mRNA, export mechanism must be used when NS1 is lacking or has not been produced at sufficient levels in early stages of infection. This is consistent with the fundamental observation that NS1 is a nonessential viral gene in cell culture whose loss nevertheless attenuates viral replication even in interferon-deficient systems (Garcia-Sastre et al., 1998). Moreover, the reduction in M1 accumulation that we observed is in line with the decreases in late gene expression noted in many previous studies of NS1 mutants (Egorov et al., 1998; Enami and Enami, 2000; Falcon et al., 2004; Garaigorta et al., 2005; Hatada et al., 1990; Salvatore et al., 2002). The low-

efficiency export of M1 mRNA in the absence of NS1 may be attributable to direct recruitment of the export apparatus through interactions with the nuclear cap-binding complex (Bier et al., 2011). Moreover, a degree of redundancy in cellular factors recruited for viral mRNA export has precedents from other viral systems (Jackson et al., 2011). Even though studying the mechanism of mRNA nuclear export in the absence of NS1 was not pursued in this dissertation, it would be an important aspect of the IAV life cycle to unveil and warrants further investigation.

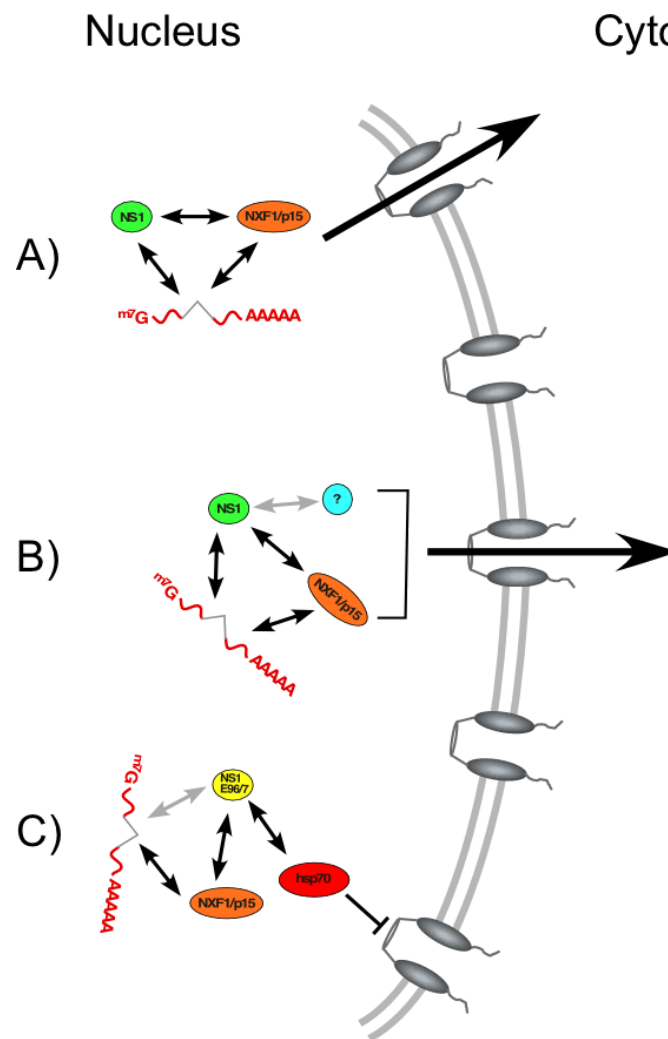


Figure 3.16 – Cartoon model synthesising results of Chapter 2. Demonstrated interactions are represented by a black arrow while hypothesised interactions are represented by grey arrows. A) Interactions shown to be required for efficient segment 7 mRNA nuclear export to occur. These interactions were identified between NS1 and segment 7 mRNA, NS1 and NXF1 and NXF1 and segment 7 mRNA via NS1. An NS1 protein capable of promoting segment 7 mRNA efficient nuclear export must support all three interactions. B) It is however possible for an NS1 mutant protein to comply with the above mentioned requisites and still be unable to carry out its export function. Hence, it is likely that an additional and unknown factor is also required for this export mechanism to be successful. C) The identification of hsp70 as interacting solely with NS1-E96/7A could shed further light onto the mRNA export mechanism as it may be the reason for segment 7 mRNA export to be blocked despite being able to support the interactions indicated in A). The block could be due to a conformational change induced by hsp70 or by the interaction masking the interaction site for another factor playing a role in this mRNA export mechanism.

Overall, in this Chapter, the role of NS1 in promoting the efficient nuclear export of segment 7 mRNA was further validated. The main findings and proposed mechanisms are depicted and summarised in **Figure 3.16**. The interactions between NXF1 and NS1, NS1 and segment 7 mRNA and NXF1 and segment 7 mRNA in the presence of NS1 were shown to be required for the efficient export of segment 7 mRNA (**Figure 3.16A**). However, since despite being necessary, those interactions were shown not to be sufficient, suggesting that NS1 must partake in further interaction(s) or recruit a further component(s) to complete its function in viral mRNA nuclear export (**Figure 3.16B**). Finally, the identification of hsp70 as the unique interacting partner of an NS1 mutant protein that could support all the studied interactions and yet was unable to promote segment 7 mRNA nuclear export (**Figure 3.16C**) should not be dismissed. It is possible that this interaction either induces a conformational change of NS1 preventing it from interacting with another factor required for export or that hsp70 outcompetes other factors for the binding site that includes mutated residues 96 and 97 of NS1.

In conclusion, we propose that NS1 acts as an adaptor protein through, but not only, its interaction with segment 7 mRNA and NXF1, the main component of the major cellular mRNA export pathway NXF1/Nxt1.

CHAPTER 4 – THE ROLE OF NS1 IN THE NUCLEAR EXPORT OF OTHER SEGMENT mRNAs

4.1 Introduction

The data described in the two previous chapters of this dissertation identified NS1 protein of IAV as a viral requirement for the efficient nuclear export of segment 7 mRNA and that in order for NS1 to be an export-competent protein: 1) both functional domains of the protein must be present; 2) NS1 must be able to interact with NXF1; 3) NS1 must be able to bind segment 7 mRNA; and 4) NS1 must be able to promote the interaction between segment 7 and NXF1. While these findings shed light on the mechanism used for the nuclear export of M1 mRNA, how the mRNAs of other IAV segments were exported remained unclear. Studies reporting that different IAV mRNAs present different requirements concerning cellular factors for their successful nuclear export suggested that segment-specific mechanisms might be in play (Amorim et al., 2007; Larsen et al., 2014; Read and Digard, 2010). For instance, when the ongoing activity of RNAP-II was identified as being required for IAV mRNA nuclear export, segment 7 transcripts were found to be more sensitive to RNAP-II inhibition than segment 5 mRNA (Amorim et al., 2007). Later, a strong dependence on NXF1 was found for the mRNAs of segments 7 and 4 while intronless, early gene mRNAs, especially segment 5, showed the least degree of dependence (Read and Digard, 2010). In addition, cell type has also been proposed to affect the extent to which NXF1 activity is required (Larsen et al., 2014). Taking these studies into consideration, it seemed unwise to generalise the data obtained in the two previous chapters across all IAV mRNAs and all cell types. These aspects were therefore addressed in this chapter; in particular the role of NXF1 in the context of segment 4 nuclear export.

4.2 Results

4.2.1 Analysis of viral replication and protein expression in cells infected with WT PR8 or NS1-N81

In chapters 2 and 3, reduced levels of M1 and M2 protein expression in the absence of intact NS1 were associated with less efficient nuclear export of segment 7 mRNA. Examination of HA accumulation in cells infected with the NS1-N81 virus (using control untreated material from a previous experiment that examined the effect of spliceostatin on segment 7 expression; **Figure 2.9**) also showed a decreased accumulation of HA but not NP (**Figure 4.1A**). Furthermore, examination of HA mRNA levels from the corresponding experiment by radioactive primer extension indicated that this was not due to lower levels of segment 4 transcription (**Figure 4.1B**). These data were therefore suggestive of a role for NS1 in enhancing HA mRNA expression post-transcriptionally; potentially at the stage of mRNA nuclear export.

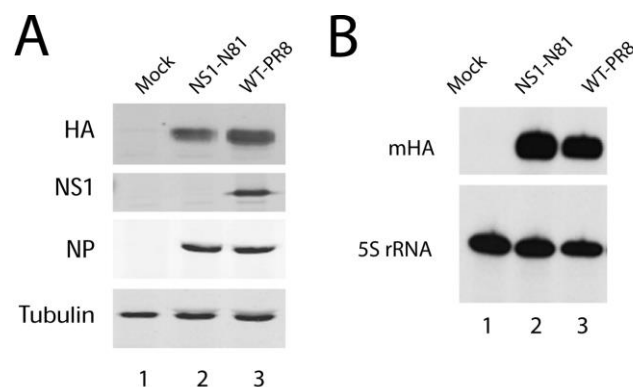


Figure 4.1 Accumulation of segment 4 gene product and mRNA in infected cells. 293T cells were mock infected or infected with WT or NS1-N81 viruses at an M.O.I. of 5. A) At 6 h p.i., cell lysates were harvested in Laemmli's sample buffer and subsequently analysed by SDS-PAGE and western blotting for the indicated proteins. B) At 6 h p.i., cells were collected and total RNA was isolated and analysed by radioactive reverse transcriptase using primers specific for segment 4 mRNA and cellular 5S rRNA, followed by urea-PAGE and autoradiography. Please note that NS1, NP and tubulin levels shown in panel A correspond to the same experiment and data presented in panel B of Figure 2.9.

However, lower levels of any or all viral proteins produced by NS1-N81 could be due to the virus replication being delayed as opposed to being completely unable to promote the efficient nuclear export of selected viral mRNAs. Since the cellular localisation of segment 7 mRNA in the context of viral infection was typically determined at 6 h p.i., it was important to compare WT and NS1-N81 growth kinetics and protein synthesis over time, beyond the 6 h p.i. time point for both WT PR8 and NS1-N81. For this purpose, single cycle growth curve

assays were performed. MDCK cells were either infected with WT PR8 or NS1-N81 at an M.O.I. of 3 and samples were collected after 1 h of adsorption (defined as 0 h p.i.) and then at various times p.i.. The titres of both viruses were determined for each time point by plaque assays. At 0 h p.i. the titre for both WT PR8 and NS1-N81 were the same, confirming that the same inoculum was used for both viruses (**Figure 4.2A**) and at 4 h p.i., the titre of both viruses were also very similar. At 6 h p.i. however, the titre determined for NS1-N81 was 10-fold lower than the one obtained for WT PR8 (**Figure 4.2**, compare red and black lines) indicating that NS1-N81 replicated more slowly compared to WT PR8. This lag increased over time and was largest at 12 h p.i. when the titre of NS1-N81 was 100-fold lower than the titre of WT PR8. While WT PR8 titres peaked at 12 h p.i., NS1-N81 virus peaked at 24 h p.i. when it registered a titre similar to WT PR8. The ability of NS1-N81 to (slowly) reach WT levels of titre could mean that the mutant virus was merely delayed in its replication kinetics and was nevertheless able to catch up over time. Alternatively, the NS1-N81 protein could have reverted to WT NS1 over time during infection, which (as explained in the Materials and Methods section) was (a) an occasional problem and (b) not surprising as only one nucleotide was changed to introduce a stop codon and produce the truncated NS1-N81 protein. However, protein analysis over time would verify if NS1-N81 was indeed delayed or if there was a reversion of the NS1 mutation, or possibly, a combination of both effects.

To this end, 293 T cells were either mock infected or infected with WT PR8 or NS1-N81 viruses at an M.O.I. of 5. Cells were harvested at 3, 4.5, 6, 8 and 10 h p.i., to cover an entire IAV replication cycle that typically takes 8 h, and analysed by western blotting.

In mock infected cells collected at 10 h p.i., only the cellular protein tubulin could be detected, confirming the lack of a viral infection throughout the entire course of the experiment (**Figure 4.2B**, lane 1). Tubulin levels were reasonably similar for both viruses at the same time points, with the exception of lower levels detected at 3 h p.i. for both viruses, most likely because of a technical error. Overall, in both viruses, it was possible to observe an increase of viral protein levels over time as would be expected during the course of an infection. NP accumulation kinetics were similar between the two viruses, while NS2 levels were higher in the NS1 mutant than in the WT virus; otherwise viral proteins were generally lower in cell infected with NS1-N81 especially at earlier times post infection before 8 h p.i.. Consistent with previous data in this thesis, M1 and M2 accumulation remained lower than in WT infection even at 10h p.i.. In contrast, expression of a polymerase protein, PB1, reached apparently normal levels by the end of the time course. HA accumulation followed a more complicated pattern, with readily detectable amounts visible at 3 h p.i., presumably reflecting detection of input material from the high MOI inoculum, as levels then declined at 4.5 h p.i..

In WT virus, amounts then increased again with a maximum at 6 h p.i., whereas the NS1-N81 virus showed more gradual accumulation kinetics in which levels slowly increased to 10 h p.i.. Analysis of NS1 expression levels in WT PR8 showed that NS1 was readily detected,

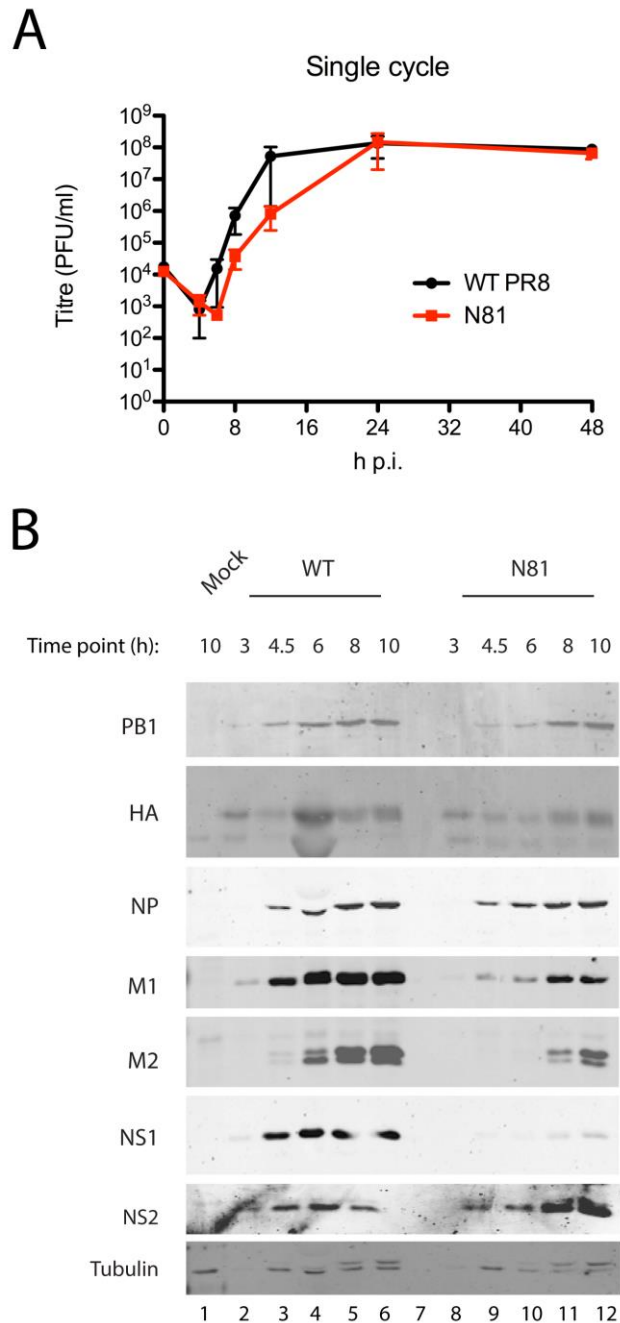


Figure 4.2 Viral kinetics and viral protein expression analysis over time. A) Growth curves of WT and NS1 mutant virus NS1-N81. MDCK cells were infected with WT PR8 or NS1-N81 viruses at an M.O.I. of 3. Supernatants were collected after 1 h of adsorption (0 h p.i.) and at 4, 6, 8, 12, 24 and 48 h p.i.. Viral titres were determined by plaque assays. Data plotted are the mean \pm range of two independent experiments. B) 293T cells were mock infected or infected with WT or NS1-N81 viruses at an M.O.I. of 5. Samples were harvested at 3, 4.5, 6, 8 and 10 h p.i. and cell lysates were analysed by western blotting for the indicated polypeptides.

even at low levels at 3 h p.i. and from 4.5 h p.i. onwards, at steady high levels, consistent with it being considered an early protein (Inglis and Mahy, 1979; Skehel, 1972). However, it was also possible to detect full-length NS1 protein in cells infected with NS1-N81, although at noticeably lower levels than WT PR8 at all time points examined. NS1-N81 mutant virus should produce a truncated and thus faster-migrating NS1 protein that lacks the effector domain. This indicated that the mutation introducing a stop codon at position 82 of NS1 was reverting during infection in this experiment. Although this reversion of the NS1-N81 mutation was not intended nor desired, the levels of NS1 were still remarkably lower when compared to WT PR8, indicating that the differences in accumulation kinetics of the various viral polypeptides could still be considered to reflect a lack of normal NS1 expression. Overall, the data suggested that a more detailed time course analysis of viral mRNA localisation was worthwhile.

4.2.2 Cellular localization of segments 7 and 5 in the presence and absence of intact NS1

Thus far in this dissertation, the cellular localisation of segment 7 mRNA was determined typically at 6 h p.i. using FISH as described previously (Amorim et al., 2007). This time point was useful and sufficient to observe shifts in the localization of segment 7 mRNA from the nucleus to the cytoplasm depending on the nature of the NS1 protein present, but nonetheless, observing the cellular localisation of viral mRNAs over time could provide further information on the timing of mRNA export and how it differed depending on whether intact NS1 was present or absent. In addition, studying the mRNA nuclear export of other IAV segments was another aim of this dissertation and, particularly, the present chapter.

To address these questions, 293T cells were mock infected or infected with WT PR8 or NS1-N81 at an M.O.I. of 5 in multiple sets. Cells were fixed at 3, 4.5, 6, 8 and 10 h p.i. before being processed for FISH using a fluorescently labelled RNA probe to detect either segment 7 or segment 5 positive sense RNA molecules. In mock infected cells, no signal was detected for segment 7 (**Figure 4.3**, red) or segment 5 (**Figure 4.3**, green) mRNAs, confirming probe specificity. As with data in the previous chapters, segment 7 mRNA was cytoplasmic in WT PR8 infection but retained in the cell nucleus in the presence of NS1-N81 at 6 h p.i.. The time course data new to this experiment showed that at 3 h p.i., segment 7 mRNA was already detectable mostly in the nucleus, both with WT PR8 and NS1-N81 viruses. However, at 4.5h the shift from nuclear to cytoplasmic localisation was evident, but not complete, in cells infected with WT PR8 while the mRNA was still markedly retained in

the nucleus in cells infected with NS1-N81. This scenario was maintained up to 8 h p.i. and was clearest at 6 h p.i.. At 10 h p.i., segment 7 mRNA was detectable in the cytoplasm of some cells infected with NS1-N81, which could be due to reversion of this mutant virus and/or slow non-NS1 dependent mRNA export. Curiously, the opposite appeared to happen in cells infected with WT PR8 at 10 h p.i., where more nuclear staining was observed, possibly due to a decreased intensity of cytoplasmic signal reflecting cytoplasmic mRNA degradation.

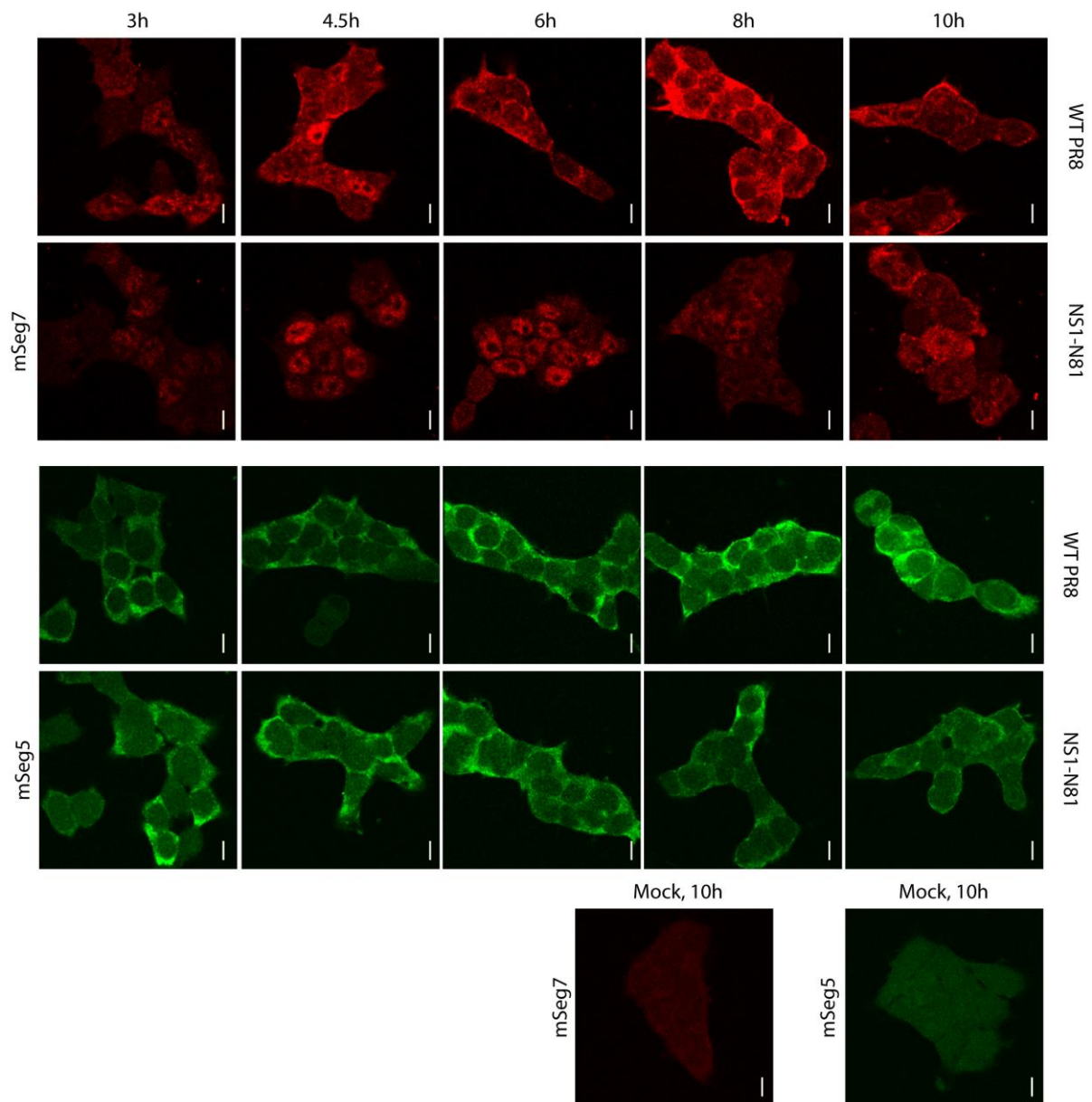


Figure 4.3 Time course of segment 5 and 7 mRNA localisation during infection. 293T cells were mock infected or infected with WT or NS1-N81 mutant virus at an M.O.I. of 5. At the specified time points, cells were fixed and processed for FISH using fluorescently labelled RNA probes for positive sense viral RNAs of segment 7 (red) or segment 5 (green). Images were captured using a Leica-TCS confocal microscope and Leica TCS analysis software. Scale-bar indicates 10 μ m.

These results further confirmed that the absence of NS1 had an impact on the mRNA localisation of segment 7 and that this effect started early in infection and was maintained throughout the majority of a full replication cycle. Analysis of segment 5 mRNA localisation showed a strikingly different outcome however. Cells infected with WT PR8 presented segment 5 mRNA clearly in the cytoplasm, from an early infection stage at 3 h p.i. up until later stages at 10 h p.i. (**Figure 4.3** green). This was expected and had been reported previously (Amorim et al., 2007; Read and Digard, 2010). However, in the presence of NS1-N81, the mRNA of segment 5 was likewise found to localise clearly in the cell cytoplasm throughout the entire course of infection suggesting that the mechanism used for the nuclear export of segment 5 mRNA was independent of the NS1 effector domain. It could thus be concluded that in line with previous studies (Amorim et al., 2007; Larsen et al., 2014; Read and Digard, 2010), different IAV mRNAs are exported using different mechanisms and have distinct requirements.

4.2.3 Cellular localization of segments 2 and 4 in the presence and absence of intact NS1

Next, segment 2 and 4 mRNA localization were examined as above. Neither segment could be detected in mock infected cells confirming probe specificity (**Figure 4.4**). Early in infection, segment 2 mRNA signal was not detected at levels above background until 4.5 h p.i. both in cells infected with WT PR8 as well as with NS1-N81 (**Figure 4.4**, red). This was not surprising even though the gene product of segment 2, PB1, is considered an early protein, given the lower levels of mRNA produced by the P gene segments (Dalton et al., 2006; Robb et al., 2009; Smith et al., 1987). Thereafter, throughout the course of infection the signal from segment 2 mRNA became more intense but remained localised mostly to the cell nucleus both in cells infected with WT PR8 as well as with NS1-N81. This could indicate that the nuclear export of segment 2 mRNA was, similarly to segment 5, independent of the presence of the NS1 effector domain. However, the apparent localisation of segment 2 RNA mostly to the cell nucleus could also be a consequence of a proportionally greater detection of cRNA, as segment 2 mRNA and cRNA were previously reported to accumulate in similar amounts (Dalton et al., 2006; Robb et al., 2009). Analysis of segment 4 cellular localisation however showed a different outcome. Segment 4 mRNA could be easily detected as early as at 3 h p.i. in cells infected with both viruses and already at that time point dissimilarities in the mRNA localisation were evident (**Figure 4.4**, green). In cells infected with WT PR8, HA mRNA was found in the cell cytoplasm and this phenotype was maintained over time. Conversely, cells

infected with NS1-N81 displayed segment 4 mRNA in the nucleus up until 6 h p.i.. Later in infection, at 10 h p.i., more cytoplasmic staining was detected in cells infected with NS1-N81 which again, could be due to reversion of the mutant virus occurring during the course of infection, and/or to slowed mRNA export kinetics. Even if that was the case, more nuclear staining was found in cells infected with NS1-N81 when compared with WT, indicating that an intact NS1 was required for the efficient nuclear export of segment 4 mRNA. It could therefore be concluded that not all IAV mRNAs share the same mechanism to achieve nuclear export. While segment 5 and possibly segment 2 mRNA nuclear export appeared to happen in an NS1-independent fashion, the requirement of NS1 for the efficient nuclear export of segments 4 and 7 was evident. Consequently, it was proposed that NS1 was necessary for the efficient nuclear export of intronless late gene mRNAs, as well as the intron-containing M1 mRNA.

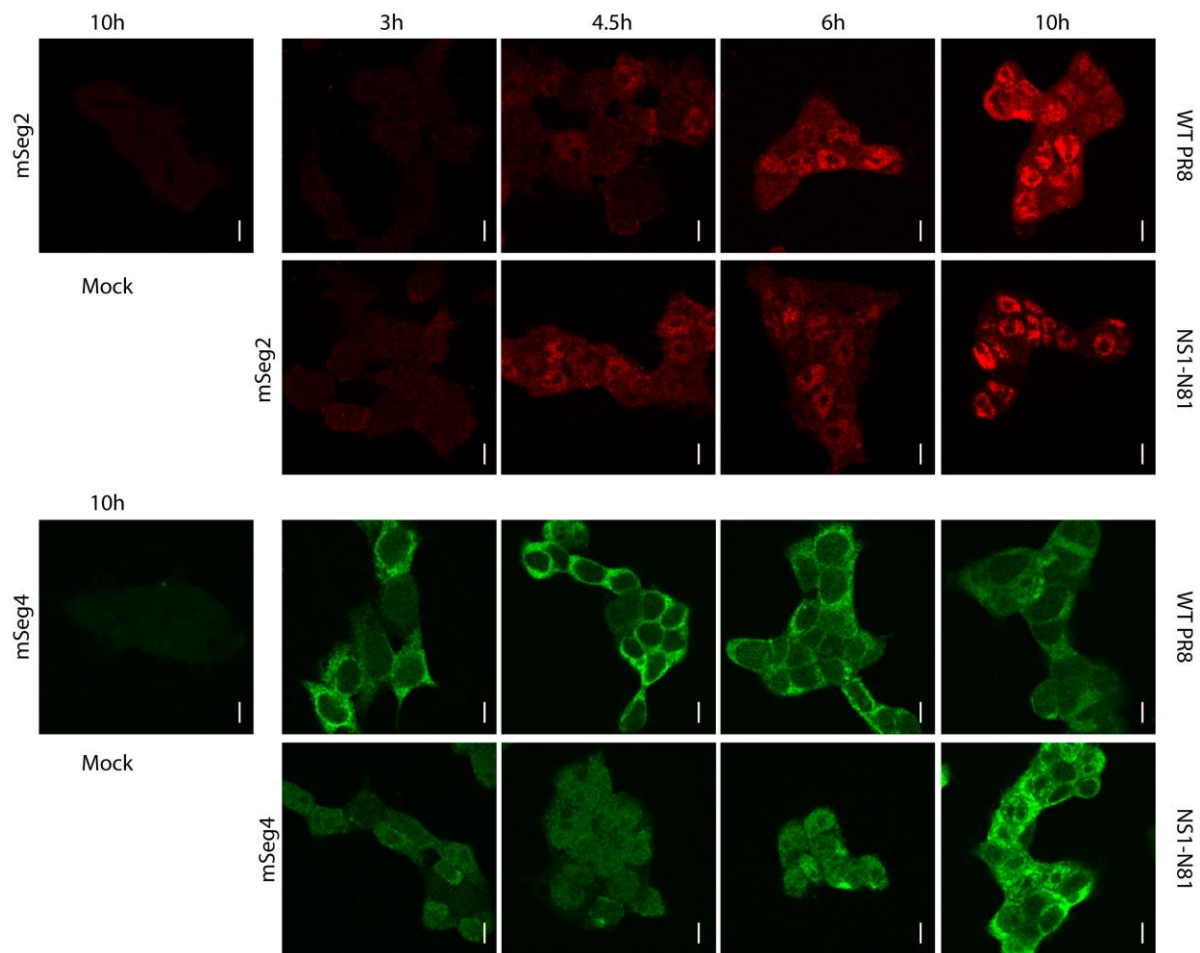


Figure 4.4 Time course of segment 2 and 4 mRNA localisation during infection.. 293T cells were mock infected or infected with WT or NS1-N81 mutant virus at an M.O.I. of 5. At the specified time points, cells were fixed and processed for FISH using fluorescently labelled RNA probes for positive sense viral RNAs of segment 2 (red) or segment 4 (green). Images were captured using a Leica-TCS confocal microscope and Leica TCS analysis software. Scale-bar indicates 10 μ m.

4.2.4 Effect of cell type on the IAV mRNA nuclear export and its dependence on NS1

The confirmation that particular IAV mRNAs utilised different mechanisms to accomplish nuclear export was broadly in line with previous studies where IAV mRNAs were found to behave dissimilarly in this respect (Amorim et al., 2007; Larsen et al., 2014; Read and Digard, 2010). Another variable that could provide more insights onto the IAV mRNA nuclear export mechanisms was the cell type. Indeed, thus far in this dissertation, the study of mRNA cellular localization was done using 293T cells. This was due to transfection-based assays being more effective in these cells and all experiments involving the minireplicon system were based on this technique. Subsequent validation of phenotypes in the context of viral infection required using the same cell type in order to avoid introducing new variables.

However, cell type has been shown to affect various processes of the IAV replication cycle, including mRNA nuclear export (Larsen et al., 2014; Lee et al., 2008). It was thus desirable to examine whether the NS1-(in)dependent localisation phenotypes of viral mRNAs seen in 293T cells would be reproducible in a different cell type. Accordingly, chicken embryo fibroblast DF-1 cells were mock infected or infected with WT PR8 or NS1-N81 viruses at an M.O.I. of 5. Cells were fixed at 6 h p.i. and processed for FISH using fluorescently labelled RNA to target mRNAs of segments 2, 4, 5 and 7. None of the viral mRNAs could be detected in the mock samples confirming probe specificity in this cell type and determining background levels of staining (**Figure 4.5**). Analysis of segment 2 cellular localisation showed the viral RNA to localise both in the nucleus as well as in the cytoplasm in the presence of WT PR8 and NS1-N81, indicating that the absence of a WT NS1 protein did not obviously alter the localisation of mRNA and/or cRNA. Equally, segment 5 mRNA displayed the same localisation irrespective of which virus the cell had been infected with. However, instead of presenting a mixed phenotype as was the case for segment 2, segment 5 mRNA was clearly located most predominantly in the cell cytoplasm, to the extent that the outlines of cell nuclei could be estimated by a void of staining in the centre of cells (**Figure 4.5**). These results were in line with the phenotypes observed for segment 5 using 293T cells (**Figure 4.3**, green) which indicated that segment 5 nuclear export occurred independently of NS1. In contrast, the cellular localisation of segment 7 mRNA further validated the results that had been thus far obtained in 293T cells, with mRNA found mainly in the cytoplasm in cells infected with WT PR8 and markedly retained in the nucleus in cells infected with NS1-N81. When examining the cellular localisation of segment 4 mRNA, a different phenotype was similarly seen depending on which virus was used for the infection assay. In cells infected with WT PR8, segment 4 mRNA was found mainly in the cytoplasm. Conversely,

cells infected with NS1-N81 displayed segment 4 mRNA mostly in the nucleus although some cytoplasmic staining could nonetheless be detected (**Figure 4.5**). In addition, the intensity of the signal was noticeably weaker in cells infected with NS1-N81, contributing to a striking difference in segment 4 localisation when compared to WT PR8 infected cells.

Taken together, these findings validated the data obtained before in 293T cells. The mRNA of both segments 2 and 5 was found to be insensitive to the lack of an export-competent NS1 protein. Moreover, and arguably more importantly, the role of NS1 in promoting the efficient nuclear export of segments 4 and 7 was demonstrated in another cell type other than 293T cells, indicating that this effect was not limited to a single cell type.

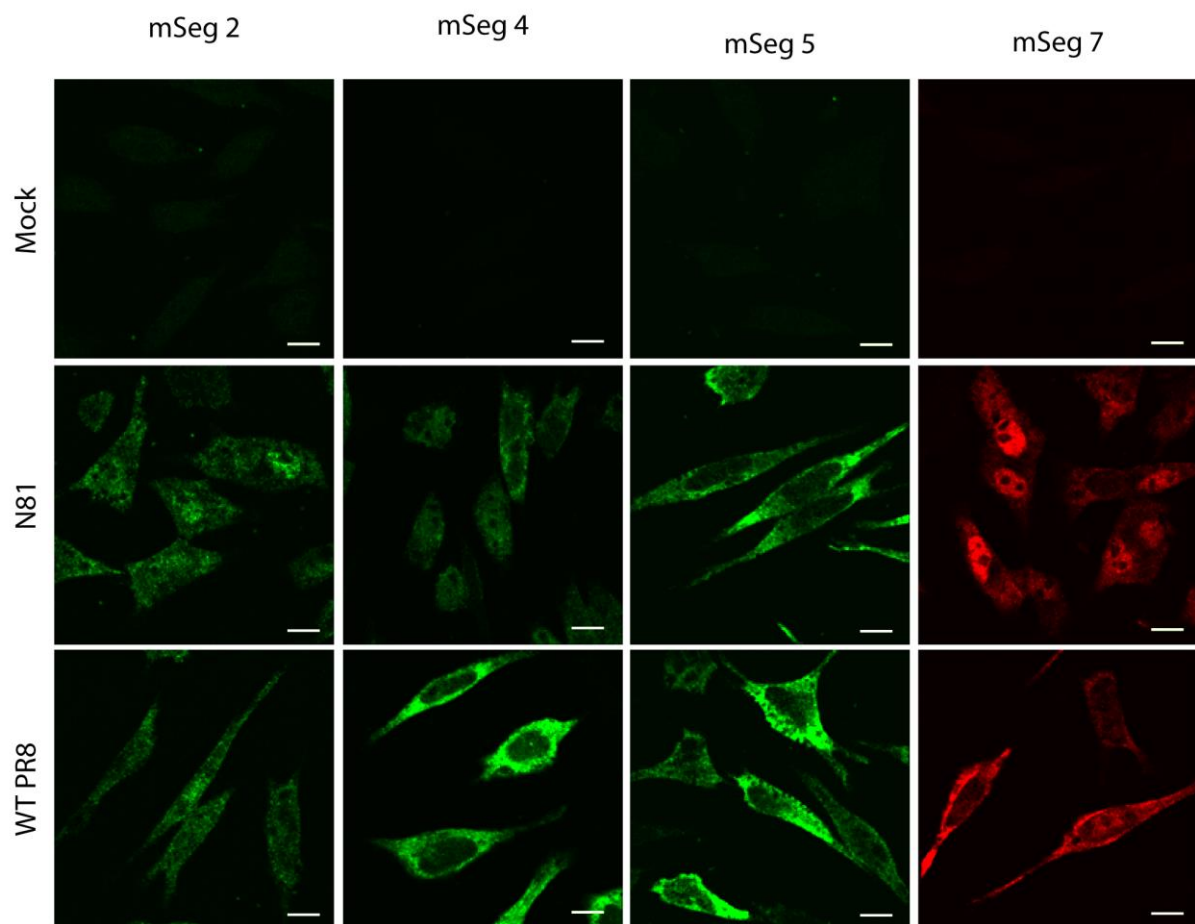


Figure 4.5 Localisation of IAV mRNAs in DF-1 cells in the presence or absence of a full-length NS1 protein. DF-1 cells were mock infected or infected with WT or NS1-N81 mutant virus at an M.O.I. of 5. At 6 h p.i., cells were processed for FISH using fluorescently labelled RNA probes for positive sense viral RNAs of the indicated segments. Images were captured using a Leica-TCS confocal microscope and Leica TCS analysis software. Scale-bar indicates 10 μ m.

Since the cellular localisation phenotypes for segments 4 and 7 were similar in regards to their dependence on an export-competent NS1 protein, it was plausible to hypothesise that these two segments used the same export mechanism. Hence it was decided to focus on segment 4 mRNA nuclear export in greater detail in an attempt to better understand its nuclear export requirements. Firstly, to further test the effect of cell type on mRNA localisation, A549 cells were mock infected or infected with WT PR8 or NS1-N81. Cells were fixed at 6 h p.i. and processed for FISH using fluorescently labelled RNA probes targeting either IAV segment 4 or 7 (**Figure 4.6**). As expected, segment 7 mRNA was found clearly to localize in the cytoplasm upon infection with WT PR8 and in the nucleus when cells were infected with PR8 NS1-N81 (**Figure 4.6**, purple). Segment 4 mRNA localisation phenotype was also as expected for the PR8 pair of viruses as it was cytoplasmic in the presence of a WT NS1 protein but nuclear in the presence of the export-incompetent truncated version of NS1 (**Figure 4.6**, red).

Overall, these results allowed validating the role of NS1 in promoting the efficient nuclear export of both segments 4 and 7 in a third cell type.

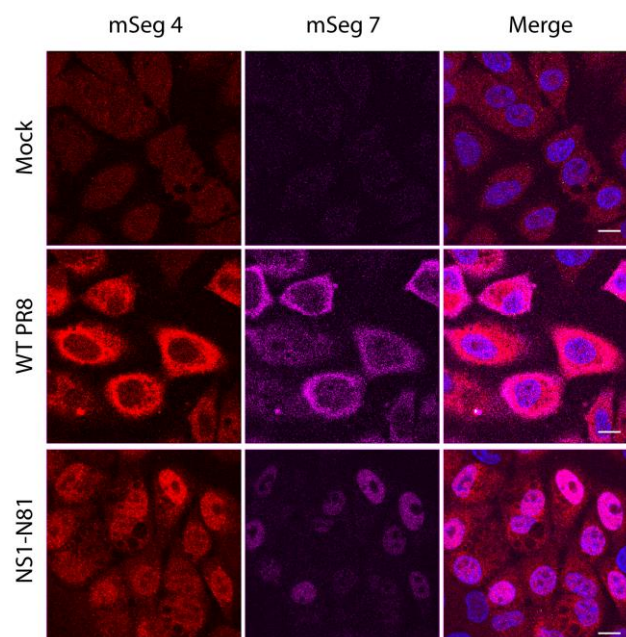


Figure 4.6 Cellular localisation of mRNAs from segments 4 and 7 in A549 infected cells in the presence or absence of a full-length NS1 protein. A549 cells were mock infected or infected with WT or NS1-N81 mutant PR8 viruses at an M.O.I. of 5. At 6 h p.i., cells were processed for FISH using fluorescently labelled RNA probes for positive sense viral RNAs of segment 4 (red) and 7 (purple) Images were captured using a Leica-TCS confocal microscope and Leica TCS analysis software. Scale-bar indicates 10 μ m.

4.2.5 Cellular localisation of minireplicon-generated segment 4 mRNA in the absence and presence of NS1

The data above suggested that both segment 4 and 7 mRNAs required NS1 for their successful nuclear export. In order to further examine if the requirements for segment 4 mRNA nuclear export matched those established for segment 7 mRNA, the minireplicon assay was used with the objective of determining the cellular localisation of segment 4 mRNA in the complete absence of NS1, as well as testing the effect of the sole addition of NS1.

To this effect, 293T cells were transfected with plasmids encoding for the three polymerase subunits, NP, a viral template for segment 4 and either EGFP, NS1-EGFP or NS2-EGFP. Cells were fixed at 24 h p.i. and processed for FISH using a fluorescently labelled RNA probe targeting segment 4 mRNA. Since the negative control lacking the PB2 polymerase subunit used in this experiment did not include EGFP, this sample was further subjected to IF targeting NP protein. The cellular localisation of the various fluorophores was then observed via confocal microscopy (**Figure 4.7A**) and cells were scored according to where segment 4 mRNA was predominantly found (**Figure 4.7B**). The 2PNP negative control sample displayed NP protein in the cell cytoplasm confirming cell transfection while segment 4 mRNA signal could not be detected (**Figure 4.7A**). In the presence of EGFP alone, segment 4 mRNA could be found retained in the cell nucleus (~50% of cells), in the cytoplasm (~30%) or both (~20%) (**Figure 4.7A and B**). Thus while the mRNA was still found to be nuclear in a substantial percentage of cells, the nuclear retention phenotype of segment 4 in the absence of NS1 was not as strong as the one observed for segment 7 (compare with **Figure 2.3**). A similar outcome was obtained when assessing the localisation of segment 4 mRNA in the presence of NS2-EGFP. In this case, a lower percentage (~20%) of cells was found to present segment 4 mRNA in the cell cytoplasm. The majority of the cells scored displayed segment 4 mRNA either in the nucleus (~50%) or both in the nucleus and the cytoplasm (~20%) (**Figure 4.7A and B**). As before, the nuclear retention of segment 4 in the presence of NS2 was not as strong or clear as the one observed with segment 7 in the presence of NS2 (**Figure 2.3**). In the presence of NS1-EGFP however, segment 4 mRNA could be found almost entirely (~95%) in the cytoplasm of individual cells, with only a small percentage of cells displaying the mRNA both in the cytoplasm as well as inside the nucleus (5%). No cells displaying segment 4 mRNA retained in the nucleus were found upon the addition of NS1-EGFP in the minireplicon setting (**Figure 4.7A and B**). Overall, even though segment 4 mRNA was found to be more cytoplasmic without the addition of NS1 than segment 7, and despite a greater variability between individual cells, a very clear shift toward cytoplasmic localisation of HA mRNA could be demonstrated upon the addition of NS1.

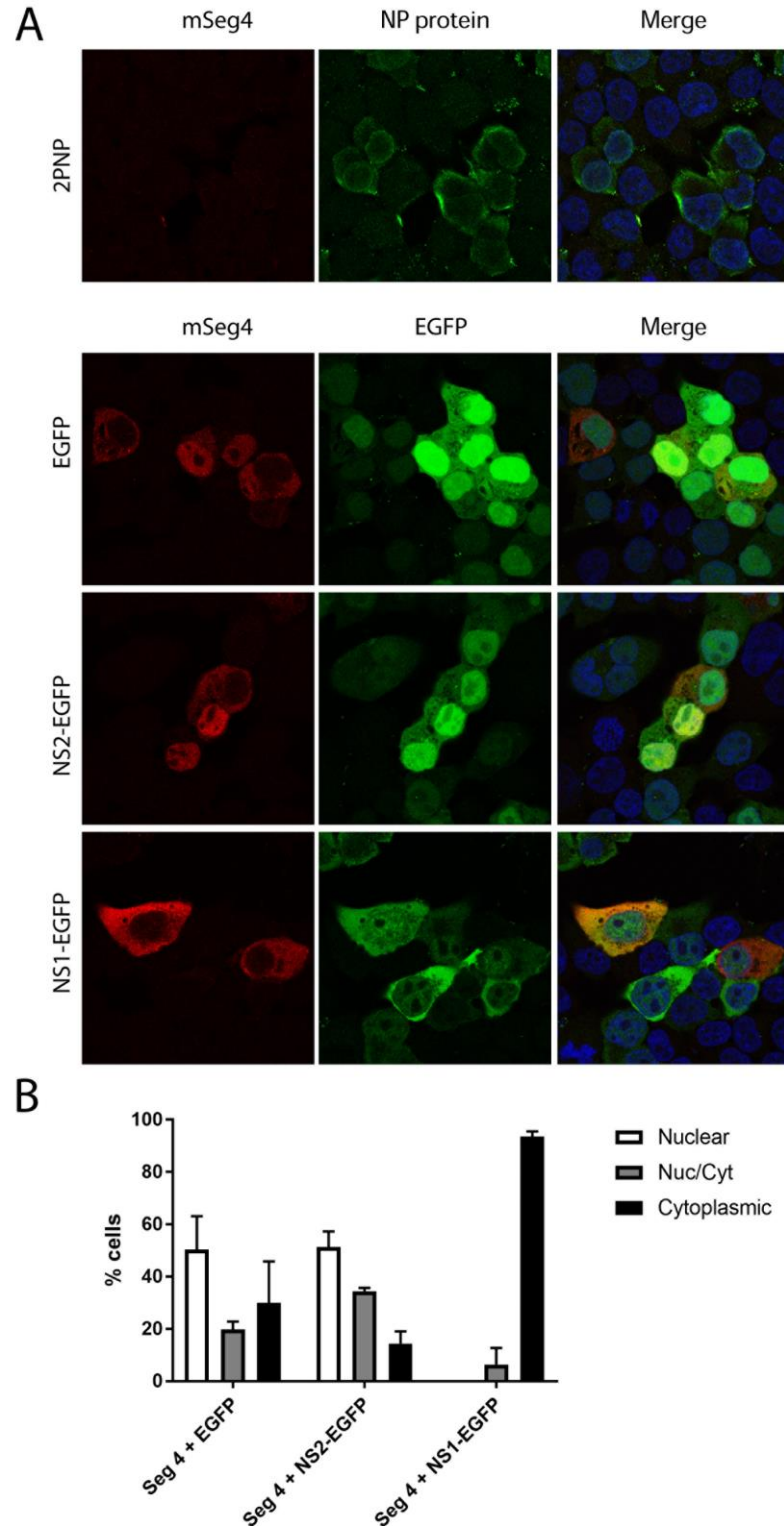


Figure 4.7 Cellular localisation of segment 4 mRNA in the presence or absence of NS1 protein in transfected cells. A) 293T cells were co-transfected with plasmids to recreate segment 7 RNPs or, as a negative control with a combination that excluded a plasmid expressing PB2 (2PNP), together with either GFP alone or GFP-tagged plasmids expressing NS1 or NS2, as indicated. Cells were incubated at 37 °C and processed for FISH analysis at 24 h p.t. using a fluorescently labelled RNA probe for the detection of positive sense viral RNAs of segment 4 (red) and GFP expression was detected by confocal microscopy (green). 2PNP transfected cells lacking GFP were stained by IF for NP (green). Images were captured using a Leica-TCS confocal microscope and Leica TCS analysis software. Scale-bar indicates 10 μ m. B) Individual cells were scored according to the predominant cellular localisation of segment 7 mRNA considering three phenotypes, nuclear, cytoplasmic or mixed. Values are the mean \pm SEM from three independent experiments.

These results confirm that segment 4 mRNA required NS1 for efficient nuclear export, although perhaps with a lower degree of NS1 dependence when compared to segment 7 mRNA.

4.2.6 Analysis of the effect of NS1 on NXF1 interaction with segment 4 mRNA

Previously in this dissertation, it was found that NS1 promoted the interaction between NXF1 and segment 7 mRNA (**Figure 3.6**). Hypothesising that both segments 7 and 4 preferentially use the same mechanism of nuclear export involving the participation of NS1, the next question to ask was whether NS1 could also facilitate an interaction between NXF1 and segment 4 mRNA.

To test this, 293T cells were transfected with GFP-NXF1 along with the minireplicon basic set of plasmids encoding PB1, PB2, PA, NP and a viral RNA template for segment 4, with or without NS1. Cells were harvested and lysed at 48 h p.t. before being subjected to GFP-Trap pull down after saving aliquots of supernatant for total RNA and western blot analysis. RNA isolation was performed and examined by primer extension. The loading control 5S rRNA was found in similar amounts in material from total cell lysates, indicating that equivalent amounts of RNA were extracted from equal numbers of cells and was not detected in the bound fractions, as expected (**Figure 4.8A**, lanes 1-6). Likewise, segment 4 vRNA could also only be detected in the total fractions, suggesting that NXF1 did not interact with this RNA species. Examination of segment 4 mRNA showed that it could not be detected in the negative control samples, confirming that the minireplicon assay was successful. While the mRNA was found in the totals fraction with or without NS1 as expected (**Figure 4.8A**, lanes 2-3), it was also clearly detected in the bound fraction, also both in the absence or presence of NS1 (lanes 5-6). More HA mRNA was precipitated in the presence of NS1 (**Figure 4.8**, lane 6). However, more segment 4 mRNA was present in the total fraction in the presence of NS1 and the difference in band intensities in the two bound fractions was not such that it would allow a clear conclusion that NS1 promoted the interaction between NXF1 and segment 4 mRNA to be made.

To complement this experiment, protein analysis of samples from the total fractions was also carried out (**Figure 4.8B**). The loading of comparable amounts of cell lysate was confirmed by the detection of equivalent amounts of tubulin. NP and GFP-NXF1 levels were also similar, indicating that transfection efficiency was equivalent in all samples. NS1 protein was detected in samples where the NS1 plasmid was included (lanes 1 and 3) but not in the sample where it was left out (lane 2). HA protein could not be detected in the 2PNP negative

control as expected (lane 1) and while it was present in cells transfected with GFP-NXF1 and the minireplicon set of proteins without NS1 (lane 2), the levels of its expression were noticeably higher in the presence of NS1 (lane 3). These results were in line with a role of NS1 in promoting the nuclear export of segment 4 mRNA as more mRNA being exported to the cytoplasm would normally result in higher amounts of protein being produced. Taken together, these data indicated that NS1 promotes the expression of HA without necessarily having a major effect on NXF1-segment 4 mRNA interactions.

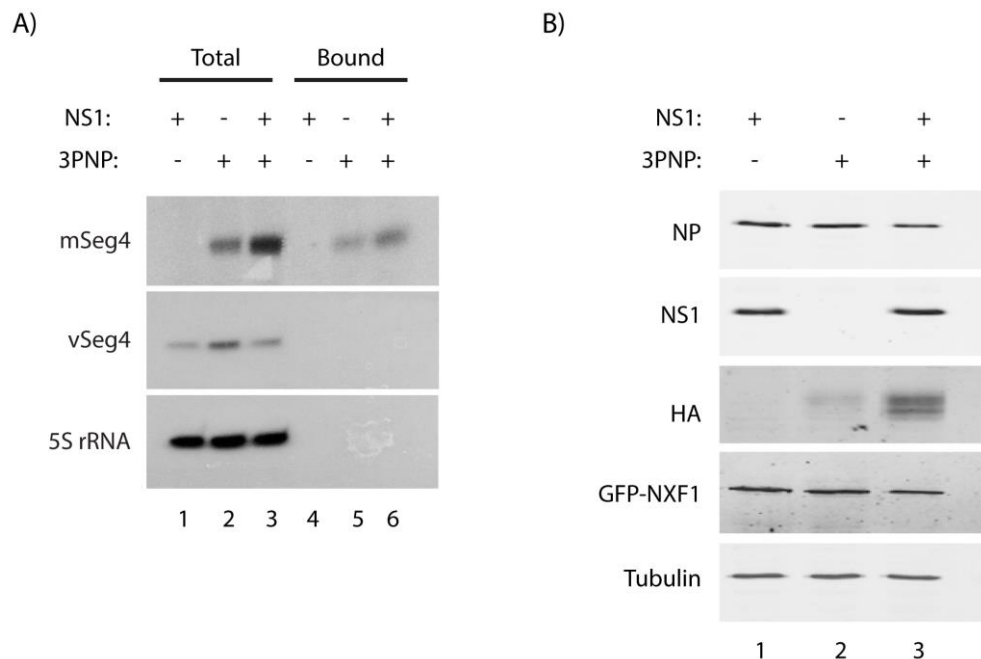


Figure 4.8 Analysis of the effect of NS1 on the interaction between NXF1 and segment 4 mRNA. 293T cells were transfected with plasmids to recreate segment 7 RNPs (3PNP+) or, as a negative control, with a 2PNP combination lacking a plasmid expressing PB2 (3PNP-) along with GFP-NXF1 and with or without NS1 as indicated. At 48 h p.t, cells were harvested and GFP-Trap pulldown assays were carried out. A) RNA was extracted from total cell lysate and GFP-Trap pulldowns (bound) and analysed by radioactive reverse transcriptase with primers specific for segment 4 mRNAs and cellular 5S rRNA followed by urea-PAGE and autoradiography. B) Aliquots of supernatant were collected prior to RNA extraction in Laemmli's sample buffer for protein analysis by western blotting for the indicated polypeptides.

4.2.7 Analysis of segment 4 mRNA localisation in the presence of NS1 mutant proteins in the context of viral infection

The realization that segment 4 mRNA efficient nuclear export involved the participation of NS1 even if the requirement was less stringent than that of segment 7 mRNA was intriguing. Earlier in this chapter it was observed that segment 4 mRNA was partially retained in the nucleus upon infection with the NS1-N81 mutant virus. This effect was shown in 293T, DF-1 and A549 cells, indicating that it was not a random phenomenon that could only be attributed to one particular cell type. Hence, the next question that was asked was

whether segment 4 mRNA nuclear export would respond in the same way as segment 7 upon infection with NS1 mutant viruses. To answer this, 293T cells were mock infected or infected with either WT PR8 or NS1 mutant viruses at an M.O.I. of 5. Cells were fixed at 6 h p.i. and processed for FISH using an RNA probe targeting segment 4 mRNA. Subsequently, cells were subjected to IF targeting the NS1 protein.

The low signal detection both for segment 4 mRNA (green) and NS1 protein (red) in the mock sample confirmed the lack of infection and set background levels (**Figure 4.9**). Conversely, strong signals from both fluorophores localised predominantly in the cell cytoplasm could clearly be detected in cells infected with WT PR8, as expected. The mutant NS1 proteins were also detected in the cell cytoplasm in all samples apart from NS1-N81, where little staining above background could be detected (**Figure 4.9**). Since the antibody used to detect NS1 was raised against the effector domain which was lacking in this mutant, this was not unexpected. After infection with NS1-N81, segment 4 mRNA was markedly retained in the host cell nuclei, confirming that nuclear export of the mRNA was not as efficient in the presence of a truncated form of NS1 protein. In cells infected with the RNA-binding domain mutants, NS1-R38A, NS1-K41A and the double mutant NS1-(R+K), mixed phenotypes were observed (**Figure 4.9**). The mRNA localisation patterns were noticeably different than that observed in WT PR8 infected cells, where all cells presented segment 4 mRNA clearly in the cytoplasm. This was not the case in cells infected with any of the RNA-binding mutants. However, there was also not such a clear nuclear retention in cells infected with these mutants as seen in cells infected with NS1-N81. Nevertheless, NS1-R38A and NS1-(R+K) showed a greater tendency to result in HA mRNA nuclear retention while a weaker effect was observed in cells infected with NS1-K41A (**Figure 4.9**). In the presence of the NS1-(S+I) mutant virus that was used as a positive control, segment 4 mRNA was displayed in the cell cytoplasm as had been observed before when studying segment 7 mRNA localisation (**Figure 3.4**). Taken together, these results were in line with the data obtained for segment 7 mRNA, where similar cellular localization phenotypes were observed when cells were infected with this same panel of NS1 mutant viruses (**Figure 3.4**). These findings support the hypothesis that NS1 is required for the efficient nuclear export of segment 4 and that both functional domains of NS1 are necessary for the protein to retain its mRNA export function.

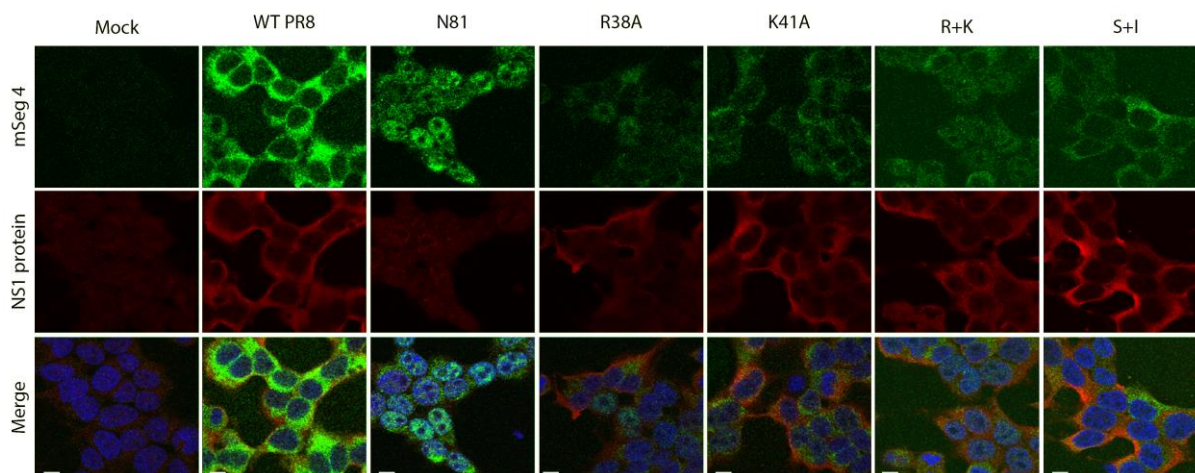


Figure 4.9 Analysis of segment 4 mRNA localisation in cells infected with NS1 mutant viruses. 293T cells were mock infected or infected with the indicated viruses at an M.O.I. of 5. At 6 h p.i., cells were processed for FISH and stained for segment 4 mRNA using a specific fluorescent RNA probe (green), then for DNA using DAPI and NS1 protein by IF (green). Images were captured using a Leica-TCS confocal microscope and Leica TCS analysis software. Scale-bar indicates 10 μ m.

4.2.8 Analysis of the interactions between segment 4 mRNA with NXF1 and NS1

In this dissertation, the study of segment 7 mRNA nuclear export showed not only that it required NS1 for its efficient export to the cytoplasm to occur, but also that NXF1 participation was necessary. It was found that if NS1 failed to: 1) interact with NXF1; 2) interact with segment 7; or 3) promote the interaction between NXF1 and segment 7 mRNA, then the nuclear export of this mRNA was inhibited (**Figures 3.5, 3.7 and 3.8**). These data lead to the hypothesis that NS1 acted as an adaptor protein between the viral mRNA and NXF1 as a way to feed the mRNA into the major cellular mRNA export pathway. The response of segment 4 mRNA to the absence of an export-competent NS1 protein was similar to segment 7, suggesting that both mRNAs utilised the same nuclear export mechanism. However, HA mRNA bound to NXF1 in the absence of NS1 and the interaction was not obviously stimulated by NS1 (**Figure 4.8**). Hence, a further attempt to understand whether successful segment 4 nuclear export could be correlated with an NS1-dependent interaction with NXF1 was made in the context of virus infection. Furthermore, the ability of mutant NS1 proteins to interact with segment 4 mRNA was also studied.

To test the effect of NS1 mutations on the interaction between NXF1 and segment 4 mRNA, 293T cells were transfected with either GFP or GFP-NXF1 and 48 h later either mock infected or infected with WT PR8 or one of the NS1 mutant viruses at an M.O.I. of 10. Cells were harvested, lysed and subjected to GFP-Trap pull down at 6 h p.i.. The RNA was isolated and examined via primer extension. Detection of 5S rRNA from aliquots of total cell lysate was similar in all samples, confirming that equivalent amounts of RNA had been extracted

and thus that samples were comparable (**Figure 4.10A**, lanes 1-8). Segment 4 mRNA was also detected in total samples except from mock infected cells, confirming that the infections were successful. Examining the bound fractions, it was found that segment 4 mRNA could be clearly detected in samples that had been infected with WT and NS1-(S+I) viruses (lanes 10 and 15, respectively) while only trace amounts were observed from cells infected with NS1-N81, NS1-R38A and NS1-K41A viruses (lanes 11-13). However, segment 4 mRNA could not be detected in the presence of the RNA-binding double mutant NS1-(R+K) (lane 14). Additionally, segment 4 mRNA was absent from a cell sample that had been transfected with GFP and infected with WT PR8 (lane 9) indicating that GFP could not precipitate segment 4 mRNA, as expected. Segment 4 mRNA could also not be detected in cells that had been transfected with GFP-NXF1 and mock infected (lane 16), confirming the specificity of the interaction observed between NXF1 and segment 4 mRNA in other samples. These results confirmed that NXF1 interacted with segment 4 mRNA as had been previously reported (Wang et al., 2008) and in contrast to the previous minireplicon-based experiment (**Figure 4.8**), suggested that the interaction between NXF1 and segment 4 is influenced by the presence of NS1, as only trace amounts of segment 4 mRNA were found to interact with NXF1 in the presence of mutant forms of NS1 unable to promote consistent cytoplasmic accumulation of the transcript. These findings are in line with what was observed for segment 7 and strengthened the adaptor hypothesis.

In order to determine whether an interaction between NS1 protein and segment 4 mRNA could be detected, 293T cells were transfected with GFP, NS1-GFP or one of the GFP-tagged NS1 mutants. At 48 h p.t., cells were infected with the NS1-N81 mutant virus at an M.O.I. of 10 to supply segment 4 mRNA and an export-incompetent NS1 in all samples so as not to mask activity of the exogenous GFP-NS1 polypeptides. Subsequently, samples were processed as described above. Equivalent amounts of total RNA were analysed for each sample as indicated by similar amounts of 5S rRNA (**Figure 4.10B**, lanes 1-8) and even though the radiograph was over-exposed on the left hand end due to leakage of the X-ray film cassette, the absence (in mock infected cells) or presence of segment 4 mRNA in similar amounts in infected with NS1-N81 mutant virus could be seen. Analysis of the bound fractions showed that segment 4 mRNA could clearly interact with WT NS1-GFP and with NS1(S+I)-GFP, both nuclear export competent proteins (lanes 11 and 16). However, in cells transfected with N81-GFP or any of the RNA-binding mutants, only trace to no detectable amounts of segment 4 mRNA were precipitated indicating that these NS1 mutant proteins could not sustain an interaction with segment 4 mRNA (lanes 12-15). Thus similarly to

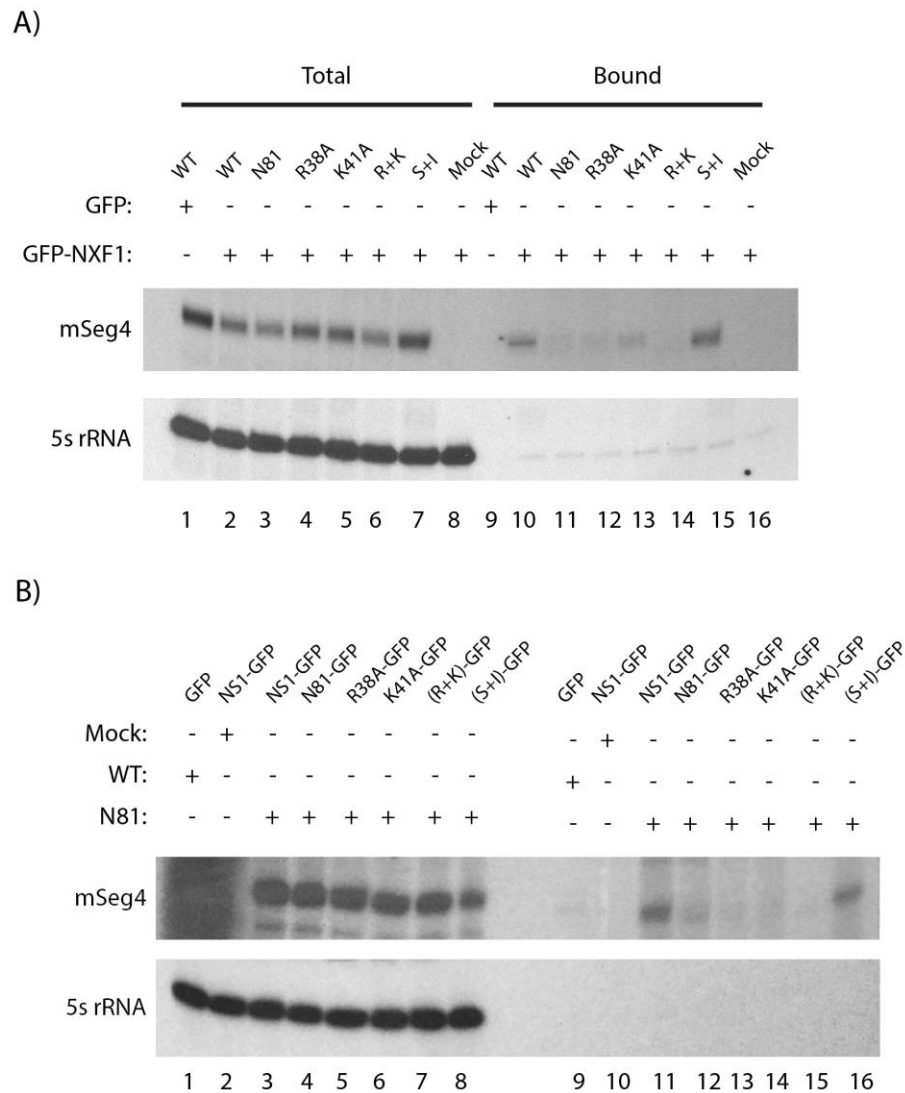


Figure 4.10 Figure 4.10 Analysis of segment 4 mRNA interactions with NXF1 and NS1. A) 293T cells were transfected with GFP-NXF1 (+) or with GFP alone (-) and 48 h later either mock infected or infected with the indicated viruses at an M.O.I. of 10, in duplicate sets. At 6 h p.i., cells were harvested and total cellular RNA was extracted from one set while the second set was subjected to GFP-Trap pulldown assays prior to total RNA extraction. RNA was analysed by radioactive reverse transcriptase with primers specific for segment 4 mRNAs and cellular 5S rRNA, followed by urea-PAGE and autoradiography. B) 293T cells were transfected with GFP-tagged plasmids expressing either GFP alone or GFP-NS1 mutant proteins, as indicated. At 48 h p.t., cells were mock infected or infected with WT or NS1-N81 as specified, at an M.O.I. of 10. At 6 h p.i., cells were harvested and total cellular RNA was extracted from one set while the second set was subjected to GFP-Trap pulldown assays prior to total RNA extraction. RNA analysis was done as described for panel A.

segment 7, segment 4 mRNA was only able to interact with NS1 proteins that were also capable of mediating efficient export of the transcript. Furthermore, it was demonstrated that both functional domains of NS1 protein were necessary as the lack of either of them resulted in the loss of the interaction. Taken together, these data indicated that indeed, the mRNA nuclear export mechanisms used by segments 4 and 7 overlap substantially and for both mRNAs, NS1 participation is required for the interaction between the viral transcripts and the cellular mRNA export machinery.

In conclusion, the findings presented in this dissertation strongly suggest that NS1 acted as an adaptor protein in order to facilitate the nuclear export of intronless, late gene mRNAs.

4.3 Discussion

The aim of the work presented in this chapter was to study the role of NS1 in the nuclear export of mRNAs from IAV segments, other than segment 7. NS1 was identified as a viral requirement for the efficient nuclear export of segment 7 in Chapter 2 and parts of the mechanism used for this were unveiled in Chapter 3. However, the immediate extrapolation and generalisation that the requirements for nuclear export would be the same for all IAV transcripts would not be sensible. For instance, the ongoing activity of RNAP-II was shown to be required for the nuclear export of segment 7 but not for segment 5 as cell treatment with DRB resulted in the nuclear retention of segment 7 while segment 5 was still found in the cytoplasm (Amorim et al., 2007). This study suggested that segment 5 mRNA used a different nuclear export mechanism than segment 7 and this was supported by further reports where nuclear export of individual IAV mRNAs presented different degrees of sensitivity to the inhibition of cellular factors involved mRNA export (Larsen et al., 2014; Read and Digard, 2010). The Read & Digard study revealed that while intronless segment 4, unspliced M1 and spliced M2 mRNAs were strongly dependent on the presence of NXF1 for nuclear export, segment 5 mRNA showed the least degree of dependency on NXF1. Similarly, reduced levels of UAP56 resulted in the inhibition of M1, M2 and NS1 trafficking and/or translation while NS2 synthesis remained unaffected. Depletion of Aly, on the other hand, showed that this cellular factor had little effect on the nuclear export of the mRNAs examined. This work concluded that not all IAV mRNAs have the same cellular requirements for their nuclear export. Furthermore, the authors proposed that a subset of mRNAs used the main cellular mRNA export pathway including most, but not all, late gene mRNAs. These findings were further supported by a later report showing that nuclear export of intronless segments 4 and 6 was reduced upon the inhibition of NXF1 while segments 1, 2, 3 and 5 remained unaffected (Larsen et al., 2014). Hence, determining whether NS1 participated in the nuclear export of all or only selected gene transcripts would contribute to increasing knowledge of the overall mechanism used by IAV to transport its mRNAs to the cytoplasm.

Since segments 1, 2, 3, and 5 are an inherent part of the minireplicon system, this approach could not readily be used to study the nuclear export of these segments. Technical

limitations were also encountered in the viral side of things, with respect to NS1 itself and the possibility of making a truly NS1-minus system. Attempts were made to rescue mutant viruses where NS1 would be completely absent or only present as a very short truncated form containing only the first 13 amino acids (Δ NS1 and NS1-N13, respectively). However, although viable viruses could be generated, it was not possible to obtain titres that would allow the use of a sufficiently high M.O.I. to successfully carry out FISH assays. Thus, the role of NS1 in the export of other IAV mRNAs was carried out using the NS1-N81 virus. This strategy had the shortcoming that the effect of the total absence of NS1 would not be possible to study. For this reason, it was relevant to determine if this mutant virus showed similar growth rates as WT or if the impaired nuclear export of segment 7 mRNA export in the presence of this mutant virus could be due to a growth defect of NS1-N81. Single cycle growth curves of WT PR8 and NS1-N81 showed that, indeed, the latter was delayed when compared to WT (**Figure 4.2A**); a result consistent with previous studies (Egorov et al., 1998; Garcia-Sastre et al., 1998; Solorzano et al., 2005). The delay was visible until 24 h p.i. at which point NS1-N81 seemed to “catch up” with WT, presenting similar titres both at 24 h p.i. and 48 h p.i.. Several of the other IAV polypeptides have functions in antagonising cellular responses to viral infection in addition to those encompassed in the NS1 RNA-binding domain, so slower rather than lower replication kinetics for the NS1-N81 mutant are not unexpected. Another possible reason for the behaviour of this virus is that on occasions, NS1-N81 was able to revert the mutation that inserted a stop codon at position 82 of the gene. Reversion of NS1-N81 was confirmed in some experiments (*e.g.* **Figure 4.2B**) by the detection of a full-length NS1 protein in western blotting analysis. This was not seen in every experiment (data not shown) and the virus was regenerated from plasmid rather than by passage of existing stocks. However, reversion of NS1-N81 could be detected as early as at 3 h p.i. when it did occur, indicating that perhaps the virus stock used in the infection already contained trace amounts of revertants which had occurred during the minimum passaging required to produce it. Nevertheless, even in this scenario, the amounts of NS1 protein produced by NS1-81 at 10 h p.i. were markedly reduced when compared to WT levels. Analysis of other viral protein expression showed that the NS1-N81 mutation changed the accumulation kinetics of most other viral polypeptides. Interestingly, NS2 protein expression increased at 8 h p.i. surpassing the levels detected for this protein in cells infected with WT virus. This is consistent with a previous report showing that inactivation of the NS1 gene increased the efficiency of segment 8 splicing, yielding increased levels of NS2 transcripts (Fortes et al., 1994). Conversely, NP, PB1, HA, M1 and M2 levels all decreased, but with the early NP and PB1 genes being least affected and the segment 7 products the most. It was

possible that decreased levels of viral proteins could be attributed to NS1-N81 being transcriptionally attenuated. Alternatively, it was also plausible that reduced viral protein expression could be the result of a defect at a post-transcriptional level. This hypothesis was supported by another experiment where more segment 4 mRNA was detected in cells infected with NS1-N81 than WT, but less HA protein was produced (**Figure 4.1A** and **4.1B**). These results indicated that HA expression was indeed attenuated at a post-transcriptional level – bearing in mind identified roles for NS1 in viral gene expression, either at the level of mRNA translation (de la Luna et al., 1995; Nordholm et al., 2017; Panthu et al., 2017; Schierhorn et al., 2017) and/or at the level of mRNA export (this thesis; (Pereira et al., 2017).

Following on from biochemical observations, the cellular localisation of mRNAs from segments 2, 5, 4 and 7 were examined over time in WT and NS1-deficient infections. As observed before, segment 7 mRNA was found in the cytoplasm of cells infected with WT PR8 and retained in the nucleus upon infection with NS1-N81 (**Figure 4.3**). Segment 7 mRNA was detected in the cytoplasm of cells infected with WT PR8 from as early as 4.5 h p.i. and was increasingly visible there until 8 h p.i. Conversely, segment 7 mRNA was retained in the nucleus during the course of NS1-N81 infection and this differential only became less pronounced at 10 h.i. by which time at least some cells contained WT NS1 due to reversion (**Figure 4.2A**). In contrast, segment 5 mRNA was readily found in the cell cytoplasm during infection with either virus, indicating that the mechanism used to export this mRNA occurred in an NS1 effector domain-independent manner. This was consistent with NP synthesis being less affected than other viral proteins in cells infected with NS1-N81 (**Figure 4.2B**). These results were also consistent with previous reports demonstrating the difference in mRNA nuclear export requirements for NP and M1 mRNAs (Amorim et al., 2007; Larsen et al., 2014; Read and Digard, 2010). Similarly, segment 2 mRNA nuclear export also did not show an obvious effect from mutating NS1 as the localisation of plus-sense RNA did not differ between cells infected with WT PR8 or NS1-N81 (**Figure 4.4**). However, since the RNA signal was found more predominantly in cell nuclei in both instances, it is possible that these results reflect a proportionally greater detection of cRNA from a segment where the two classes of positive-sense RNA are present in similar amounts (Dalton et al., 2006; Robb et al., 2009). A higher detection of nuclear cRNA could mask a potential effect of NS1-N81 on mRNA localisation, so the possibility that NS1 plays a role in segment 2 efficient mRNA export cannot be excluded. However, the levels of cytoplasmic staining seen in WT PR8 infected cells were not noticeably diminished in cells infected with NS1-N81 which suggests that the mRNA export of segment 2 mRNA is indeed independent

of the NS1 effector domain. In contrast, the absence of WT NS1 had a clear impact on the localisation of segment 4 mRNA (**Figure 4.4**). While cells infected with WT PR8 displayed segment 4 mRNA in the cytoplasm throughout the course of the infection, a clear mRNA retention phenotype could be observed in cells infected with NS1-N81, at least at earlier times p.i.. These findings indicated that NS1 played a role and was required for the rapid cytoplasmic accumulation of segment 4 mRNA. The mRNA localisation phenotypes for all these segments were further validated as they were maintained in a different cell type, DF-1 cells upon infection with either WT PR8 or NS1-N81 (**Figure 4.5**). In these cells the nuclear retention of segments 4 and 7 in the absence of a full-length NS1 protein was evident as was the insensitivity of segment 5 mRNA. Segment 2 mRNA on the other hand seemed to be more nuclear in cells infected with NS1-N81. This could indicate that NS1 is potentially more important for nuclear export of this mRNA in some cell types. Indeed, it has been suggested that cell variability influences the mRNA export requirements of IAV mRNAs (Larsen et al., 2014). In this report, segment 5 mRNA export was shown to be sensitive to inhibition of NXF1 in 293T cells but not in A549 cells. A549 cells were used in this Chapter, but only to study segment 4 and 7 mRNAs. Since the Larsen *et al.* report was published after the end of the experimental work presented in this dissertation the nuclear export of segment 5 in A549 cells was not examined, as its independence from NS1 had been clearly shown both in 293T and DF-1 cells. However, it would be interesting to test whether segment 5 mRNA could be affected by the absence of WT NS1 in A549 cells in future studies.

Even though the minireplicon system was not suitable for studying nuclear export of segments 2 and 5 mRNAs, it could be used to study segment 4 as the latter gene is not a required component of the system. Additionally, using the minireplicon assay to study segment 4 mRNA nuclear export allowed a test of the effect of the total absence of NS1. Addition of NS1 to the segment 4 minigenome system resulted in a clear increase in cytoplasmic detection of segment 4 mRNA (**Figure 4.7**), in agreement with the results obtained for segment 7 mRNA in chapter 2. However, nuclear retention of segment 4 mRNA in the total absence of NS1 was not as pronounced as the one observed for segment 7. This was visible as an increased percentage of cells displaying a mixed phenotype of segment 4 mRNA both in the nucleus and cytoplasm. The reasons for this difference are not clear since in Chapter 2, results obtained in the context of viral infection were corroborated by assays performed using the minireplicon system. Nevertheless, the addition of NS1 resulted in a clear shift of segment 4 mRNA towards cytoplasmic localisation, further establishing NS1 as a viral requirement for the efficient nuclear export of segment 4 mRNA. The similarities in

behaviour of segments 4 and 7 mRNA to perturbations of nuclear export have been noted previously (Amorim et al., 2007; Read and Digard, 2010). Both transcripts were previously shown to strongly depend on the presence of NXF1 for cytoplasmic accumulation (Read and Digard, 2010). This dependence was also observed in Chapter 2 and was attributed to the ability of NXF1 to interact with export-competent NS1 proteins and to segment 7 mRNA via NS1. Hence it was plausible to conjecture that the NS1 interaction with NXF1 and segment 4 mRNAs were also required for the efficient nuclear export of segment 4 mRNA. However, using the minireplicon system, it was found that the interaction between NXF1 and segment 4 mRNA was readily detectable even in the absence of NS1 (**Figure 4.8A**). Nevertheless, HA protein expression was strikingly higher when NS1 was present, which is in line with NS1 facilitating the nuclear export of segment 4 mRNA (**Figure 4.8B**). Since the results obtained using the minireplicon assays seemed to be less clear for the study of segment 4, the panel of NS1 mutants was used instead in order to better evaluate the overlaps between segments and 7 and 4 mRNA nuclear export mechanisms.

Upon infection with the various NS1 mutant viruses, segment 4 mRNA exhibited similar phenotypes to M1 mRNA. HA mRNA was found in the cytoplasm of cells infected with WT PR8 and NS1-(S+I) and retained (to greater or lesser extents) in the nuclei of cells infected with NS1-N81 and the RNA-binding domain mutants NS1-R38A, NS1-K41A and NS1-(R+K) (**Figure 4.9**). This indicated that, as with segment 7, both functional domains of NS1 were required to promote efficient cytoplasmic residency of segment 4 mRNA. The interactions between NXF1 and segment 4 mRNA in the presence of NS1 mutants were also much like the behaviours seen with segment 7. In the context of virus infection, NXF1 binding to segment 4 mRNA was notably stronger in the presence of export-competent NS1 proteins (**Figure 4.10A**). Likewise, the interaction between NS1 and segment 4 mRNA was found to only be supported by NS1 proteins also capable of promoting the efficient nuclear export of segment 4 mRNA (**Figure 4.10B**). These findings indicate that only NS1 mutants able to promote the interaction between NXF1 and segment 4 mRNA can retain their viral mRNA nuclear export function. Additionally, the participation of NS1 in the efficient nuclear export of intronless segment 4 mRNA further strengthens the results obtained in Chapter 2 concerning the splicing independence of the mechanism. Indeed, since segment 4 mRNA is not spliced, NS1-mediated mRNA export does not necessitate mRNAs undergoing splicing events, contrary to what was proposed in a recent report (Mor et al., 2016).

Taken together, the results presented and discussed in this Chapter make a strong case for segments 4 and 7 utilising the same mRNA nuclear export mechanism; one involving NS1

participation in order to introduce them into the major cellular mRNA export pathway. The suggestion that different export mechanisms are used according to IAV mRNA categorisation into classes such as intronless, spliced, unspliced, early or late is not novel (Amorim et al., 2007; Larsen et al., 2014; Read and Digard, 2010; Wang et al., 2008). However, the data from this Chapter further support this separation in that NS1 is required for the nuclear export of HA and M1 mRNAs, which are considered late class and unspliced mRNAs.

While it would have been very useful to include data on segment 6 mRNA export, since this is also a late and intronless segment, attempts to obtain a working FISH probe to determine its cellular localisation were unsuccessful. Additionally, the lab's lack of a specific antibody against this protein also prevented inferences to be made by protein expression analysis. Nevertheless, previous studies have provided evidence that does point towards segment 6 using the same mRNA nuclear export mechanism as segments 4 and 7. Specifically, segment 6 mRNA was found to colocalise with NS1 in the cell nucleus in addition to be shown to interact with both NS1 and NXF1 (Wang et al., 2008). Furthermore, segment 6 mRNA nuclear export was found to be reduced upon inhibition of RNA Pol II (Vogel et al., 1994) or NXF1 (Larsen et al., 2014).

Thus, we conclude by proposing a model for a sub-set of IAV mRNAs nuclear export in which NS1 acts as an adaptor protein between viral late gene intronless/unspliced mRNAs and NXF1 as a strategy to incorporate viral mRNAs into the major cellular mRNA export pathway. We further speculate that this mechanism could be used as a kinetic regulator in the IAV life cycle to ensure that specific mRNAs are exported at appropriate times in infection.

CHAPTER 5 – CONCLUDING REMARKS

This dissertation focused on the study of IAV mRNA nuclear export in an attempt to identify and determine the requirements needed for this important step of the virus life cycle to occur.

In previous work carried out in the Digard laboratory, Dr. Eliot Read remarked that, while segment 7 mRNA could be found in the cell cytoplasm during viral infection using FISH, this mRNA was retained in the nucleus in a minireplicon assay. This observation was the basis for the work described in this dissertation, starting from the postulation that a viral component absent from the minireplicon system could play a role in the mRNA export of segment 7 mRNA. Thus, Chapter 2 reports on the study of viral requirements for an efficient nuclear export of segment 7 mRNA. Using FISH as the technique to observe the mRNA cellular localisation allowed the identification of NS1 as the viral component in the absence of which, most segment 7 mRNA was found to be markedly retained in the cell nucleus. Furthermore, it was demonstrated that the presence of a poly(A) tail in the mRNA was another requirement for nuclear export and NS1 presence could not override the lack of this signal. Lastly, the potential link between mRNA export and splicing was explored and it was demonstrated that mRNA nuclear export enabled by NS1 ensued independently of splicing events.

The work presented in Chapter 3 examined in more detail the role of NS1 in promoting the efficient export of segment 7 mRNA focusing on NS1 protein itself and its connection with the major cellular mRNA export pathway, NXF1/Nxt1. This study was carried out using a set of NS1 proteins carrying mutations in several sites previously associated with many of the function attributed to NS1. Both functional domains of NS1, the RNA-binding domain and the effector domain, revealed to be necessary for the protein to retain its nuclear export function. Moreover, in order for nuclear export to be successful, NS1 further needs to interact with NXF1 protein and segment 7 mRNA. Importantly, NS1 must also be capable of promoting the interaction between NXF1 and segment 7 mRNA. Any NS1 mutant protein examined that failed to support either one of these interactions also failed to promote efficient export of segment 7 mRNA.

However, and surprisingly, even while these requirements were established as necessary, they were found to be insufficient. Indeed, analysis of NS1 protein with mutations inserted in the effector domain showed that even though these proteins could interact with NXF1 and promote the interaction between NXF1 and segment 7 mRNA, the nuclear export of the latter was nevertheless impaired. This suggests that the role of NS1 in the mechanism of mRNA nuclear export is not limited to its interaction with segment 7 mRNA and NXF1 and that it is required for a further step in the export pathway, upstream of the participation of NXF1. Based on the findings presented in this Chapter it is possible to conjecture a model for segment 7 mRNA nuclear export where NS1 would bind the mRNA and recruit an unknown cellular factor which would in turn recruit NXF1. NS1 would then interact with NXF1 and transfer segment 7 mRNA to NXF1 as a mechanism to feed the viral mRNA onto the main cellular mRNA export pathway leading to its successful export to the cell cytoplasm.

Chapter 4 focused on studying the mRNA nuclear export of IAV segments other than 7 and the potential involvement of NS1 in this process. The nuclear export of mRNAs from segments 2 and 5 is insensitive to the lack of NS1 and suggests that the transcripts utilise a different mechanism to achieve nuclear export. However, segment 4 mRNA presents essentially the same requirements as segment 7 mRNA. Efficient nuclear export of segment 4 mRNA occurs only when NS1 is present and capable of interacting with NXF1 and the mRNA while also having to be able to promote the interaction between segment 4 mRNA and NXF1. Since the common features shared by segments 4 and unspliced segment 7 are their classification as late mRNAs that evade splicing, it is proposed that the role of NS1 is restricted to this class of viral transcripts. It is reasonable to hypothesise that the participation of NS1 in the nuclear export of late transcripts could act as a time regulation ensuring these mRNAs are more efficiently exported later in infection, when higher levels of NS1 would have been produced.

Nevertheless, the results presented in this dissertation raise several important questions. Since NS1 is suggested to play another role in the export mechanism other than its interaction with NXF1 and viral mRNAs, it is plausible that NS1 could recruit another cellular factor involved in the NXF1/Nxt1 pathway, but which one? The most likely candidates would be components of the hTREX complex as they have a similar role of interacting with cellular mRNAs and recruiting NXF1. Another key question raised by results described in Chapter 4 concerns the nuclear export of early and intronless mRNAs. What is the mechanism used by these transcripts to reach the cytoplasm? There is mounting evidence

that not all IAV mRNAs have the same requirements for nuclear export (Amorim et al., 2007; Larsen et al., 2014; Read and Digard, 2010). While the mechanism for late intronless mRNAs appears to be getting clearer with the previous identification of NXF1 pathway, and now NS1, being required, the question of how early intronless transcripts are exported remains largely unknown.

Given the nonessential nature of NS1 in the life cycle of IAV, how do late intronless mRNAs get exported to the cytoplasm in the absence of NS1? This is perhaps the most intriguing question raised by the results presented in this dissertation. It is important to note that even when mRNA nuclear retention was noticeable, gene product synthesis from the mRNA was reduced, but not totally inhibited. Nuclear translation is one possibility (Dolan et al., 2010). However, it is also possible that a redundant, and less efficient (or slower), mechanism exists that is independent of NS1. Such mechanism would ensure that sufficient amounts of mRNA are exported to allow protein synthesis in levels that would permit the replication of the virus.

Overall, the findings presented in this dissertation provide new insights into how late and intronless IAV transcripts are efficiently exported from the cell nucleus to the cytoplasm. Specifically, while a role for NS1 in viral nuclear export had been suggested, hinted at and hypothesised before, the data here provided the most concrete and direct evidence up to date in support of this hypothesis. The data provided a deeper understanding and characterized the role of NS1 in mRNA nuclear export mechanisms in greater detail. We showed that, in order to promote mRNA export, NS1 needed to be able to interact with NXF1 and the viral mRNA. Moreover, it was demonstrated that NS1 promoted the interaction between NXF1 and viral mRNAs. These results support and strengthen the hypothesis that NS1 acts as an adaptor protein between the viral mRNA and the nuclear export cellular machinery. Finally, this study reinforces the argument that different segments vary in their strategy to achieve mRNA nuclear export raising the intriguing question of what mechanism is used for the export of early and intronless mRNAs.

CHAPTER 6 – MATERIALS & METHODS

6.1 Materials

6.1.1 General reagents

General purpose reagents (analytical grade if available) were supplied variously by Bethesda Research Laboratories (BRL), BDH Chemicals Ltd., VWR, Fisons and Sigma. The following reagents were obtained from the indicated companies:

Acrylamide/bis-acrylamide (37:5:1)	GeneFlow
Agarose	Boehringer Mannheim
Blocking reagent (for FISH)	Roche
Dimethyl sulphoxide (DMSO)	Aldrich Chemical Company Ltd.
Lipofectamine 2000	Invitrogen
Nucleobond Xtra Midi kit	Machery Nagel
Nitrocellulose	Schleicher and Schuell Co.
Opti-MEM	Invitrogen
Protein molecular weight markers	New England Biolabs
QIAquick PCR purification kit	Qiagen
SV Miniprep kit	Promega
Trizol reagent	Invitrogen
X-ray film (Kodak X-Omat S)	Kodak Ltd.

6.1.2 Radiochemicals

γ [³²P] ATP (3000 Ci/mmol)

Perkin Elmer

6.1.3 Enzymes

DNA restriction endonucleases were supplied by Boehringer Mannheim, Invitrogen and New England Biolabs. Alternatively, the following enzymes were obtained from the companies indicated:

AMV Reverse Transcriptase	Promega
<i>Pfu</i> native DNA polymerase	Stratagene
PNK (T4 Polynucleotide Kinase)	Fermentas
RNaseOUT™ Recombinant Ribonuclease Inhibitor	Invitrogen
SP6 RNA polymerase	Roche
Superscript III Reverse Transcriptase	Invitrogen
T7 RNA polymerase	Promega

6.1.4 Oligonucleotides

Oligonucleotides were ordered from Sigma-Genosys unless otherwise indicated.

Table 6.1 Primers used for primer extension

Primer	Purpose	Sequence
PD62	Segment 5 mRNA detection	5' CGT CCA ATT CCA CCA ATC 3'
PD64	Segment 5 vRNA detection	5' GTC TTC GAG CTC TCG GAC G 3'
PD376	Segment 2 mRNA detection	5' GTA TCC TGT TCC TGT CCC 3'
PD378	Segment 8 mRNA detection	5' TTA GGG ATT TCT GAT CTC GGC 3'

PD380	Segment 4 mRNA detection	5' TCA CTG TCA CAT TCT TCT GCA GC 3'
PD381	Segment 4 vRNA detection	5' CCC TGG GGG CAA TCA GTT TCT 3'
PD1156	Segment 7 mRNA detection	5' GAA GGC CCT CCT TTC AGT CC 3'
PD1157	Segment 7 vRNA detection	5' GAA AAG AGG GCC TTC TAC GG 3'
PD1158	5S rRNA detection	5' TCC CAG GCG GTC TCC CAT CC 3'
PD1162	Segment 6 vRNA detection	5' TGG ACT AGT GCG AGC AGC AT 3'
PD1163	Segment 6 mRNA detection	5' TCC AGT ATG GTT TTG ACT TCC 3'

Table 6.2 Primers used for the addition of restriction sites to allow subcloning of NS1 and NS2 genes into pEGFPN1 vector

Primer	Purpose	Sequence
PD1081	Reverse transcription reaction to have cDNA from segment 8 in order to clone NS2 into the vector	5' TTT TTT TTT TTT TTT AGT ACT A 3'
PD1082	Addition of <i>KpnI</i> restriction site to 5' end of segment 8	5' GCA ACC <u>GGT ACC</u> ATG GAT CCA AAC ACT GTG TC 3'
PD1083	Addition of <i>AgeI</i> restriction site to 3' end of NS1 coding region	5' GGT TGC <u>ACC GGT</u> ACT TCT GAC CTA ATT GTT CC 3'
PD1084	Addition of <i>AgeI</i> restriction site to 3' end of NS2 coding region	5' GGT TGC <u>ACC GGT</u> ATA AGC TGA AAC GAG AAA GTT C 3'

Table 6.3 Primers used for the subcloning of segment 7 intron sequence into a pcDNA3 vector to make an M1 specific RNA probe for FISH

Primer	Purpose	Sequence
PD1088	Addition of <i>HindIII</i> restriction site to 5' end of segment 7 intron sequence	5' GCA ACC <u>AAG CTT</u> GTA CGT ACT CTC TAT CAT CCC GTC 3'
PD1089	Addition of EcoRI restriction site to 3' end of segment 7 intron sequence	5' GGT TGC <u>GAA TTC</u> CTG CAA ATT TTC AAG AAG ATC AT 3'

6.1.5 Immunological reagents

Table 6.4 Antibodies against influenza A proteins

Antibody target	Source/Reference	Application (dilution)
Rabbit polyclonal anti-PB2	(Amorim et al., 2007)	WB (1:500)
Rabbit polyclonal anti-PB1 (V19-7)	(Digard et al., 1989)	WB (1:500)
Rabbit polyclonal anti-NP (A2915)	(Noton et al., 2007)	WB (1:500), IF (1:500)
Rabbit polyclonal anti-M1	(Amorim et al., 2007)	WB (1:500)
Mouse monoclonal anti-M2	Clone 14C2, Abcam	WB (1:1000)
Rabbit polyclonal anti-NS1 (V29)	(Carrasco et al., 2004)	WB (1:500)
Rabbit polyclonal anti-NS2	Kind gift of Dr Augustin Portela	WB (1:500)
Rabbit polyclonal anti-PR8	(Amorim et al., 2007)	WB (1:500)
Anti-NS1	Kind gift of Professor E. Fodor	WB (1:5000)

Table 6.5 Antibodies against cellular proteins

Antibody target	Source	Application (dilution)
Rat monoclonal anti- α tubulin	YL1/2; MCA77G AbD-Serotec	WB (1:1000)
Mouse monoclonal anti-Aly	Clone 11G5 from Abcam	WB (1:500)
Mouse monoclonal anti-UAP56	Ab47955 from Abcam	WB (1:500)
Mouse monoclonal anti-TAP/NXF1	Clone 53H8 Abcam	WB (1:200)
Mouse monoclonal anti-Hur/ELAV1	Clone 19F12 from Abcam	WB (1:200)
Mouse monoclonal anti-Nup62	BD Biosciences 610497	WB (1:500)
Mouse monoclonal anti-Hsp70	BD Biosciences 610607	WB (1:500), IF (1:500)

Table 6.6 Other primary antibodies

Antibody target	Source	Application (dilution)
Mouse monoclonal anti-GFP (J1-8)	Clontech 632380	WB (1:5000)
Mouse monoclonal anti-V5 tag	Clone V5.E10 from abm	WB (1:500)

Table 6.7 Secondary antibodies

Antibody	Source	Application (dilution)
IRD800 conjugated goat anti-mouse	Licor 926-32210	WB (1:10000)
IRD800 conjugated donkey anti-rabbit	Licor 926-32213	WB (1:10000)
IRD680 conjugated goat anti-rabbit	Licor 926-32221	WB (1:10000)

IRD680 conjugated donkey anti-mouse	Licor 926-32222	WB (1:10000)
Alexa 680 conjugated goat anti-rat	Invitrogen A-21096	WB (1:10000)
Alexa 633 conjugated goat anti-rabbit	Invitrogen A-11034	IF (1:1000)

Additionally, the DNA-binding fluorescent dye DAPI (4',6'-diamidino-2-phenylindole hydrochloride; Sigma) was used where indicated at 1:5000.

6.1.6 Plasmids

Table 6.8 List of plasmids used

Plasmid	Details
pPolIHA	Plasmid expressing PR8 segment 4 vRNA sense sequence from a polI promoter (de Wit et al., 2004)
pPolINP	Plasmid expressing PR8 segment 5 vRNA sense sequence from a polI promoter (de Wit et al., 2004)
pPolIM	Plasmid expressing PR8 segment 7 vRNA sense sequence from a polI promoter (de Wit et al., 2004)
pPolINS	Plasmid expressing PR8 segment 8 vRNA sense sequence from a polI promoter (de Wit et al., 2004)
pPolIMU	Plasmid expressing PR8 segment 7 vRNA sense sequence from a polI promoter with the wildtype oligoU stretch at the 5' end mutated into a oligoA stretch (Read, 2010)
pcDNA:PB2	Mt. Sinai PR8 segment 1 in a pcDNA3 backbone (Invitrogen); (Mullin et al., 2004)
pcDNA:PB1	Mt. Sinai PR8 segment 2 in a pcDNA3 backbone (Invitrogen); (Mullin et

	al., 2004)
pcDNA:PA	Mt. Sinai PR8 segment 3 in a pcDNA3 backbone (Invitrogen); (Mullin et al., 2004)
pcDNA:HA	Mt. Sinai PR8 segment 4 in a pcDNA3 backbone (Invitrogen); (Mullin et al., 2004)
pcDNA:NP	Mt. Sinai PR8 segment 5 in a pcDNA3 backbone (Invitrogen); (Mullin et al., 2004)
pcDNA:M1	Mt. Sinai PR8 segment 7 in a pcDNA3 backbone (Invitrogen); (Amorim et al., 2007); a kind gift of Dr D. Elton
pCB8+	A cDNA copy of segment 8 from the Mt. Sinai strain of PR8 cloned into plasmid pSP65 (Promega). A kind gift of S. Inglis
pCB8-	A cDNA copy of segment 8 from the Mt. Sinai strain of PR8 cloned into plasmid pSP65 (Promega). A kind gift of S. Inglis
pCAGSNS2	Influenza A NS2 expression vector in a pCAGGS backbone; a gift from W. Barclay
pCAGSNS1A	Influenza A NS1 expression vector in a pCAGGS backbone; a gift from W. Barclay
pCAGSNS1B	Influenza B expression vector in a pCAGGS backbone; a gift from W. Barclay
pGFP-NXF1	GFP tagged NXF1 expression vector; a gift from A. Whitehouse (Williams et al., 2005)

6.1.7 Bacterial strains

DH5 α

6.1.8 Virus strains

H1N1 A/PR/8/34 An MDCK cell adapted version of the National Institute for Biological Standards and Control strain of PR8 rescued from plasmids (de Wit et al., 2004)

6.1.9 Mutant viruses

The following mutant viruses were made by Dr. Helen Wise, using the H1N1 A/PR/8/34 background.

Table 6.9 List of mutant viruses used

Mutant virus	Mutation	Expected effect of the mutation
NS1-N81	Stop codon at position 81	Stop codon introduced at position 82. The mutant virus produced a truncated form of NS1 comprising the N-terminal 81 a.a. and most of the effector domain is eliminated (Zurcher et al., 2000)
NS1-R38A	R38A	Inhibition of the RNA-binding domain by residue substitution at position 38 (Wang et al., 1999)
NS1-K41A	K41A	Inhibition of the RNA-binding domain by residue substitution at position 41 (Wang et al., 1999)
NS1-(R+K)	R38A, K41A	Inhibition of the RNA-binding domain by residue substitution at positions 38 and 41 resulting in a double mutant (Wang et al., 1999)
NS1-(S+I)	F103S, M106I	Restoration of the CPSF binding ability that the PR8 strain does not possess (Kochs et al., 2007)
NS1-E96/7A	E96A, E97A	Blocks the ability of NS1 to suppress TRIM25-mediated RIG-I signal transduction resulting in the

		inhibition of host IFN response (Gack et al., 2009)
NS1-Y89A	Y89A	Inhibits the ability of NS1 to activate the PI3K pathway (Aramini et al., 2011)
NS1-Y89F	Y89F	Inhibits the ability of NS1 to activate the PI3K pathway (Hale et al., 2006)
NS1-W187R	W187R	Prevents NS1 dimerization via its effector domain (Hale et al., 2008a)

6.1.10 Drugs

Spliceostatin was a gift from M. Yoshida (Kaida et al., 2007) and was used at a concentration of 100 ng/ml

6.1.11 Eukaryotic cells

293T	A derivative of human embryonic kidney 293 cells expressing the SV40 Tumour antigen; epithelial cell line; (DuBridge et al., 1987)
A549	Polarised human lung epithelial cell line (Giard et al., 1973)
MDCK	Madin-Darby Canine Kidney; polarised epithelial cell line (Gaush et al., 1966)
DF-1	Chicken fibroblast cell line (Himly et al., 1998)

6.1.12 Media and solutions

Eukaryotic tissue culture media

Dulbecco's modified Eagle's medium (DMEM) and Opti-MEM were obtained from PAA or Invitrogen. Complete medium: DMEM was supplemented with foetal calf serum (FCS) (10% v/v), 200 mM glutamine (final concentration), 100 U/ml penicillin and 10 µg/ml streptomycin.

Serum-free medium: FCS was omitted from the mixture of complete medium.

Virus growth medium: 1 µg/ml trypsin (Worthington Biochemical Corporation) and 0.14% bovine serum albumin (BSA; Sigma) were added to serum free medium.

Bacterial culture media

2TY	10 g/l tryptone, 10 g/l yeast extract (OXOID), 5 g/l NaCl
2TY amp	2TY supplemented with 100 µg/ml ampicillin
2TY kan	2TY supplemented with 25 µg/ml kanamycin
2TY agar	2TY amp or 2TY kan with 20 g/l agar

Other solutions and buffers

(i) For competent cells

CCMB	10 mM CH ₃ COOK, 10 mM MgCl ₂ .6H ₂ O 80 mM CaCl ₂ .2H ₂ O, 10% v/v redistilled glycerol. 20 mM MnCl ₂ .4H ₂ O
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(ii) Agarose and primer extension gel electrophoresis

TBE (1x)	90 mM Tris-HCl, 90 mM boric acid, 2.5 mM EDTA
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(iii) Nucleic acid loading solutions

DNA loading solution (10x)	50% (v/v) glycerol, 100 mM EDTA, pH 8.0, 1% SDS, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol
RNA loading solution	98% formamide, 10mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue

(iv) Acrylamide gels

6 % Urea Acrylamide	7 M Urea, 6% (w/v) acrylamide:bis-acrylamide (19:1), 1x TBE; add 100 µl 10% APS (w/v) and 10 µl TEMED per 10 ml fresh before pouring gels
SDS-PAGE mix	10% acrylamide resolving gel 3.3 ml acrylamide/bis-acrylamide (30:1), 2.5 ml 1.5 M Tris-HCl pH 8.8, H ₂ O to 10 ml, 100 µl APS 10% (w/v), 10 µl TEMED
4 % acrylamide stacking gel	1 ml acrylamide/bis-acrylamide (30:1), 1 ml 1 M Tris-HCl pH 6.8, 5 ml H ₂ O, 100 µl APS 10%, 10 µl TEMED

(v) Protein solutions

Laemmli's sample buffer	20% (v/v) glycerol, 2% (w/v) SDS, 62.5 mM Tris-HCl, 100 mM DTT, 0.01% (w/v) bromophenol blue
PAGE running buffer	25 mM Tris-base, 200 mM glycine, 0.1% (w/v) SDS
Western blot transfer buffer A	0.3 M Tris-base, 20% methanol
Western blot transfer buffer B	25 mM Tris-base, 20% methanol
1x Fix	50% methanol, 10 % acetic acid
Coomassie stain	0.2% Coomassie Blue, 10% acetic acid, 50% ethanol

(vi) Pulldown buffers

TRAP lysis buffer	50 mM Tris-HCl pH 8, 25% glycerol, 0.5% IGEPAL CA-630, 0.1% Triton X-100, 200 mM NaCl, 1 mM DTT, 1 mM PMSF, 1x protease inhibitor cocktail (Roche)
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TRAP dilution buffer	10 mM Tris-HCl pH 8, 0.1% IGEPAL CA-630, 150 mM NaCl, 0.5 mM EDTA, 1 mM PMSF, 1x protease inhibitor cocktail
(vii) FISH buffers	
SSC (20x)	3 M NaCl, 0.3 M sodium citrate, pH 7
Pre Hybridisation buffer	10 mM EDTA, 35 mM NaH ₂ PO ₄ ·H ₂ O, 60% formamide, 2X SSC, 5% dextran sulfate, 250 ng/ml tRNA
Buffer 1	100 mM Tris-HCl pH 7.5, 150 mM NaCl
Buffer 2	100 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mg/ml blocking reagent
(viii) Others	
PBS	138 mM NaCl, 2.7 mM KCl, 8 mM Na ₂ PO ₄ , pH 6.7
PBS-formaldehyde	PBS containing 4% (v/v) formaldehyde 37% (Sigma)
PBS-T	PBS containing 0.1% Tween 20 (Sigma)
PBS-PMSF	PBS containing 1% PMSF (Sigma)

6.2 Methods

6.2.1 Bacterial work

Preparation of frozen competent bacterial cells

An overnight culture of *E.coli* strain DH5 α (5 ml) was used to inoculate a 2TY culture at 1:100 and was incubated at 37°C with shaking for approximately 3 h. The bacterial culture was harvested in a pre-chilled centrifuge at 6,000 rpm for 10 min at 4 °C. The supernatant was decanted and the cells were gently resuspended in 1/4 of starting volume of CCMB. After incubation on ice for 4 h the bacteria were pelleted as described before and the supernatant was decanted. 1/12 of the starting volume CCMB was used again to resuspend the bacteria. Then the bacteria were dispensed into 200 μ l volumes in 0.5 ml microcentrifuge tubes and snap-frozen in dry ice mixed with methanol. The aliquots of frozen competent bacteria were then stored at -70 °C until required for transformation.

Transformation of competent bacterial cells

DH5 α cells were thawed on ice. Typically, 50 μ l of cells were added to 50 ng of DNA to be transformed, the mix gently flicked and incubated on ice for 30 min. The cells were heat shocked in a 42 °C water bath for 45 sec and transferred to ice for 5 min. The cells were then plated in pre-warmed 2TY agar plates with the appropriate antibiotic. When transforming kanamycin resistant plasmids, 1 ml 2TY without antibiotics was added to the cells and cells were incubated at 37 °C for 1 h with shaking for recovery prior to plating.

6.2.2 DNA assays

Preparation of plasmid DNA

Small scale DNA preparation was carried out using Wizard SV Minipreps DNA Purification System (Promega) according to the manufacturer's instructions.

Large scale DNA preparation was performed using a Nucleobond Xtra Midi kit (Macherey-Nagel), again according to the manufacturer's instructions.

DNA concentration was determined using a Nanodrop spectrometer and the purity estimated by the OD₂₆₀ to OD₂₈₀ ratio.

Polymerase Chain Reaction (PCR)

i) Reverse transcription

For reverse transcription of an RNA sequence prior to sequencing or standard PCR amplification the RNA sample was mixed with 4 µl of the required primer (10 µM) and 2 µl of MQ-H₂O. The mix was heated to 70 °C for 5 min then allowed to cool in ice. The transcription mix was then added (5 µl 5x AMV RT buffer, 1 µl 10 mM dNTP mix (dATP, dCTP, dGTP, dTTP), 0.5 µl (5 U) AMV reverse transcriptase and MQ-H₂O to make a final volume of 25 µl and the reaction mixed. The samples were incubated for 60 min at 42 °C and then moved to ice. In order to avoid any carryover of non-inactivated enzyme, the samples were processed using a PCR purification kit (QIAGEN) to remove the enzyme.

ii) PCR

For cloning of viral sequences into the pEGFP-N1 vector, the following strategy was applied. The fragment of interest was amplified using 5' and 3' terminal forward and reverse primers with additional endonuclease restriction sites by PCR. 50 µl PCR reaction mixtures containing up to 100 ng of DNA template, 200 nM of each primer, 1x Pfu buffer, 200 µM each dNTP mix (dATP, dCTP, dGTP, dTTP; Roche) and 2.5 U Pfu DNA polymerase were prepared. Initially, DNA was denatured for 2 min sec at 94 °C. Then for each cycle, the DNA was denatured at 94 °C for 60 sec, primers annealed at an appropriate temperature (based on the T_m value of the oligonucleotides), usually around 50 °C for 60 sec and elongated at 72 °C for 2 min/Kb of plasmid. This cycle was repeated 30 times, following which the reactions were incubated a further 10 min at 72 °C, and cooled to 4 °C. Amplified DNA products were processed as described below for insertion into target plasmids.

Restriction enzyme digestion

DNA was digested for 1 hour with 3-5 units (per µg of DNA) of the appropriate enzyme. Enzymes were used in the manufacturer's recommended buffer and digestion was carried out at the recommended temperature.

Agarose gel electrophoresis

Agarose gels were prepared by dissolving 0.8-1 % w/v in TBE by heating. After cooling the molten agarose for approximately 15 min ethidium bromide was added at a final concentration of 0.5 µg/ml. Once completely set, gels were immersed in TBE and the DNA samples loaded in DNA loading buffer and migrated at 10 V/cm until individual bands were resolved.

Visualisation of DNA

Agarose gels were visualised by UV transillumination after staining with ethidium bromide using a Gene Genius Bio-imaging System (Syngene) with GeneSnap GelViewer software.

Isolation of DNA fragments from agarose gels

Following digestion with restriction enzymes and fragment separation by electrophoresis, the fragments were visualized by UV transillumination and the required fragments were excised using a clean scalpel. Agarose slices (typically 100 µl) were transferred to a 1.5 ml microfuge tube and DNA was extracted using Qiagen Gel Extraction Kit according to manufacturer's instructions.

Ligation of DNA fragments

Isolated DNA of digested vector or insert DNA fragments were mixed at a molar ratio of approximately 1:3 of vector to insert. Vector and insert were covalently linked by incubating 1h at room temperature with 400 U of T4 DNA ligase (New England Biolabs) in a final volume of 20 µl containing 1x T4 ligase buffer. As a negative control, insert was omitted from the ligation reaction, and 2 µl of the ligation reactions were used to transform 20 µl of competent *E.coli*.

6.2.3 Eukaryotic cell tissue culture

Passaging and seeding cells

All cell types were grown in complete medium at 37 °C at 5% CO₂ and passaged when cells became confluent. Spent media was removed and the monolayer washed once in PBS.

Trypsin (10 ml 0.05% trypsin, 0.02% EDTA in /PBS) was added and cells were incubated until they detached (approximately 2 min for 293T cells, 5 min for A549 and DF-1 cells and 15 min for MDCK cells). Trypsinisation was stopped by adding 10 ml complete medium, cells were spun down at 1200 rpm for 2 min and resuspended in fresh complete medium at 1:10.

For seeding cells, a 10 µl sample was taken from the fresh resuspension and cells were counted using a haemocytometer (Weber Scientific International) to determine cell density. Cells were then diluted in order to obtain the appropriate density and seeded onto fresh tissue culture flasks or wells.

Coating with poly-D-lysine

The adhesion of 293T cells to plastic surfaces is weak and coating was necessary for FISH and IF; procedures that require cells to remain attached after several washing steps. In these cases, 13 mm coverslips were placed into wells of a 24 well plate and 400 µl poly-D-lysine (Sigma, 5 mg/ml) in PBS added to each well. After a period of approximately 15 min the coverslips were washed three times with PBS prior to seeding.

6.2.4 Transfection of Eukaryotic cells

293T cells transfections were carried out using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, cells were seeded at the appropriate density (see Table 6.10) one day prior to transfection. Lipofectamine 2000 was mixed with Optimem and left for approximately 5 min while the DNA to be transfected was diluted in Optimem. The lipofectamine 2000/Optimem mix was added dropwise to the DNA/Optimem mix and left for approximately 20 min. In the meantime, the medium on cells was replaced by Optimem. Finally the lipofectamine 2000/DNA mix was added dropwise evenly into each well and the transfected cells left for 24 or 48 hr before further experimental procedures. When cells were left for 48 hr the Optimem medium on cells was replaced by complete medium 24 hours post-transfection.

For experiments that required cells to be transfected with more than one plasmid the total amount of DNA was equally divided by the number of plasmids to be used.

Table 6.10 Transfection conditions for 24 and 6 well plates

Per well	24 well plate	6 well plate
Cell density	$1-2 \times 10^5$	1×10^6
Total DNA (μg) in ... μl of Optimem	0.8 in 50	2-4 in 250
Lipofectamine 2000 (μl) in ... μl of Optimem	1 in 50	5 in 250
Transfection volume (μl)	100	500
Final volume in the well after transfection (ml)	1	1.5

6.2.5 Viral assays

All work involving unfixed virus was carried out in a containment level 2 environment.

Generation of P0 viral stocks

Reverse genetics viruses were generated using the PR8 system developed by the Fouchier laboratory (de Wit et al., 2004). For each well of a 6-well plate, 4 μl of lipofectamine 2000 was added to Optimem medium for a final volume of 100 μl . The lipofectamine/Optimem mix was incubated at room temperature for 5 min. 0.25 μg of each pDUAL rescue plasmid was suspended in a final volume of 100 μl Optimem per well. The lipofectamine/Optimem mix was combined with the DNA/Optimem mix after the initial incubation and left for 30 min at room temperature. During this incubation, 293T cells were harvested, counted and diluted to a concentration of 1.4×10^6 cells/ml in complete medium. 1 ml of diluted 293T cells was then aliquoted into each well and 200 μl of DNA/lipofectamine/Optimem transfection mix added and mixed. The transfected cells were then moved to a designated virus handling area and incubated overnight at 37°C. The next morning, the medium was carefully removed and replaced with 2 ml of virus growth medium, and the cells were then left for a further 48 h. After noting the appearance of cells and media, transfected cells were then scraped into their media and pelleted by centrifugation at 3000 rpm for 5 min. The supernatants were aliquoted to provide the P0 virus stock while the cell

pellets were lysed in 250 µl Laemmli's sample buffer and boiled for 10 min to allow confirmation of viral gene expression by Western blotting.

Generation of P1 viral stocks

To generate passage 1 (P1) viral stocks in MDCK cells, T25 tissue culture flasks were seeded with MDCK cells and grown until to confluency. The cells were infected with 100 µl of P0 virus stock in 900 µl serum free medium (corresponding to a multiplicity of infection of between 0.0001 and 0.01) and returned to the incubator for 1 h. The inoculum was then overlaid with an additional 6.5 ml of virus growth medium and incubated for approximately 48 h at 37°C. The supernatant was harvested, clarified by centrifugation (2600 rpm for 10 min at 4°C) and subsequently aliquoted and stored at -70°C.

Generation of egg-grown viral stocks

Embryonated chicken eggs were inspected by candling on day 9-10 of gestation, and eggs lacking a well-developed embryo discarded. The border of the air sac and the allantoic cavity (at the blunt end of the egg) was marked with pencil on the outside of the shell, at a position opposite the embryo. On day eleven of gestation, each egg was inoculated with ~1000 PFU (or the maximum possible number of PFU for the highly defective mutants) of virus in 100 µl total volume of PBS. Inoculation was performed with a 16 mm, 25 gauge needle, injecting vertically downwards through the air sac and into the allantoic cavity, on the opposite side of the egg to the embryo. Prior to inoculation, the surface of the egg was cleaned by wiping with ethanol; afterwards the puncture in the shell was sealed with invisible tape. The eggs were then incubated at 37°C, tilting every 30 min for 48 h, and then transferred to 4°C on day 13, a Home Office approved Schedule 1 technique.

Allantoic fluid was then harvested from eggs as follows. Each egg was placed with the blunt end upright and the tape removed. Forceps were used to break away the egg shell and membranes above the air sac, and the membranes overlying the allantoic cavity were peeled back. A 10 ml pipette was used to extract allantoic fluid, with forceps used to keep the mouth of the pipette clear of membranes and other debris. Eggs were discarded if the yolk had ruptured during harvesting of the allantoic fluid or if an egg was showing obvious signs of bacterial contamination. Allantoic fluid from eggs with replicate infections was pooled, and clarified by centrifugation at 2500 rpm for 10 min at 4°C. Mock-infected allantoic fluid was prepared in a similar fashion. Samples of allantoic fluid were aliquoted and stored at -70°C.

Due to reversions encountered occasionally with the NS1-N81 mutant virus, viral stocks were not passaged but obtained by re-rescuing the mutant virus each time.

Infection of Eukaryotic cells

Spent media was removed from cells and cells were washed once with 1ml SFM. The appropriate amount of virus was added to SFM according to the desired M.O.I. to obtain an inoculum with a final volume of 200 μ l or 400 μ l for 24 or 6 well plates, respectively. Plates were gently agitated and cells were incubated at 37 °C at 5 % CO₂. 30-45 min later cells were overlaid with 2 ml viral growth medium for virus titration or 1 or 2 ml complete medium for 24 or 6 well plates, respectively, for further analysis. Cells were again incubated as above for the time required for each experiment.

Plaque assays

MDCK cells were seeded in 6 well plates (1×10^6 cells per well) and incubated under standard cell conditions overnight. A 10-fold dilution series was generated for each virus and typically the 10^{-3} to 10^{-8} dilutions were used for titration. Cells were washed once in SFM and infected with 400 μ l of each dilution, each dilution corresponding to a different well. Cells were overlaid with 2 ml of viral growth medium diluted 1:1 with 2.4% Avicell microcrystalline cellulose (Avicell RC581; FMC BioPolymer; (Matrosovich et al., 2006)) in dH₂O and plates were incubated under standard cell conditions for 48 hr.

Cells were then fixed in 4 % formaldehyde in PBS for 20 min and washed once in water before being stained with approximately 2 ml 0.1 % toluidine in dH₂O blue for 10 min. Cells were then washed three times in water and dried before plaques were counted. Typically wells with a number of plaques ranging from 50 to 100 were chosen for counting. It was assumed that each plaque corresponded to one plaque forming unit and the viral titre was determined in pfu/ml.

Growth curves

Growth curves were obtained by infecting the indicated cell type ($\sim 2 \times 10^5$ cells per well of 24 well dish) with a low (MOI 0.005) or high (MOI 3-5) multiplicity of infection in a total volume of 200 μ l in SFM. After 1 h at 37 °C, all medium was removed and cells being infected for single cycle growth curves were washed for 1 min in acid buffer (150mM NaCl, 10mM HCl, pH 3) to remove input virus before overlaying with 1 ml viral growth medium.

After incubation at 37 °C until the desired time points post infection, supernatant was harvested, spun down (3000 rpm for 5 min in a Beckman Allegra 6R centrifuge) and aliquoted for titration by plaque assay.

6.2.6 Immunofluorescence assays

Fluorescence in situ hybridization (FISH)

(i) Viral m- and vRNA specific probes synthesis – FISH probes

FISH probes were synthesized essentially as described in (Amorim et al., 2007). Briefly, 5-10 µg of plasmid DNA was linearised with the appropriate restriction enzyme and purified using a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. The linearised DNA was eluted in 20 µl MQ-H₂O.

Table 6.11 Plasmids and enzymes list used to generate specific viral RNA probes

Plasmid	Target segment	mRNA probe		vRNA probe	
		Enzyme for linearisation	Enzyme used for transcription	Enzyme for linearisation	Enzyme used for transcription
pcDNA:PB2	1	KpnI	SP6	XbaI	T7
pcDNA:PB1	2				
pcDNA:PA	3				
pcDNA:HA	4				
pcDNA:NP	5				
pcDNA:M1	7	HindIII		EcoRI	
pcDNA:M1(52-739)	7 (intron)				
pCB8+	8	-	-	XbaI	SP6
pCB8-	8	XbaI	SP6	-	-

The linearised plasmid was used as template for the intended probe in a transcription reaction that includes the components added in the order listed below.

Table 6.12 Components used in the transcription reactions to generate FISH probes

Component	Amount used per reaction
MQ-H ₂ O	Up to a final volume of 20 µl
Plasmid (linearised)	1 µg
Transcription buffer (5x)	4 µl
0.1 M DTT	1 µl
RNaseOUT	1 µl
rATP, rCTP, rGTP (10 mM)	1 µl of each
rUTP (100 µM)	2.4 µl
Alexa 488/Cy3/Cy5 labelled UTP (100 nmol)	2.5 µl

To the above listed components, 2 µl of the appropriate polymerase was added and the reaction was incubated for 2 hr at either 40 °C or 37 °C for SP6 or T7 polymerase reactions, respectively. 1 µl DNaseI was then added and the reaction incubated at 37 °C for 15 min. The components listed below were then added to the reaction.

Table 6.13 Components added to the transcription reaction for FISH probes generation

Component	Amount used per reaction
EDTA (0.5 M)	1 µl
LiCl (4 M)	20 µl
Salmon sperm DNA(SSDNA), Promega, (10 mg/ml)	20 µl
MQ-H ₂ O	40 µl

200 µl 100% EtOH was added and the mix was kept at -70 °C overnight. The mix was then centrifuged at 13000 rpm at 4 °C for 5 min and the supernatant discarded. The pellet was washed three times, each time with the addition of 1 ml 70% EtOH followed by centrifugation as before. The pellet was dried for 10-15 min at room temperature and finally resuspended in 100 µl of a mix containing 96.5 µl Tris pH 8 (10 mM), 2.5 µl RNaseOUT and 1 µl DTT (0.1 M). The pellet was left for 10 min at room temperature and the dissolved RNA probe aliquoted in volumes of 20 µl and stored at -70 °C.

(ii) FISH assay

Cells seeded onto coverslips and either transfected or infected were removed from the incubator at the appropriate time and washed once in PBS for 5 min on a rocker at room temperature. Cells were fixed with 4% formaldehyde in PBS for 20 min and washed twice with 500 µl PBS for 5 min. 25%, 50% and 75% EtOH solutions were added to the cells sequentially for 5 min each and cells were left in 100% EtOH for 30 min at 4 °C for dehydration. Cells were rehydrated by reversing the process and adding sequentially solutions of 75%, 50% and 25% for 5 min each. Cells were washed twice in PBS for 5 min, permeabilised with 0.2% Triton X100 in PBS for 5 min and again washed twice in PBS for 5 min. Cells were post-fixed in 1% formaldehyde in PBS for 10 min, washed twice in PBS for 5 min and incubated with 400 µl pre-hybridisation buffer for 2 hr at 37 °C on a rocker. During this time, 1.5 µl of the appropriate FISH probe was added to 400 µl of fresh pre-hybridisation buffer. The mix was vortexed for 2 seconds, boiled for 5 min, cooled on ice and then 1 µl RNaseOUT was added to the mix. After the 2 hr incubation period, the pre-hybridisation buffer was replaced with 500 µl of the FISH probe mix and cells were left overnight at 37 °C in a humidified chamber. Cells were washed three times in 60% formamide/2x SSC in MQ-H₂O for 15 min at room temperature and then twice in PBS for 5 min. Cells were washed once in buffer 1 for 2 min and blocked with buffer 2 for at least 30 min at room temperature. 1/10000 DAPI in PBS was added to the cells for 2 min and cells were washed three times in PBS for 5 min before being mounted. Slides were kept at 4 °C until being imaged.

Immunofluorescence after FISH

Following the FISH assay described above cells were incubated with 200 µl of PBS containing the desired primary antibody for 1 hr at room temperature with rocking. Cells were washed three times in PBS for 5 min before being incubated with 200 µl of PBS containing the appropriate secondary antibody for 45 min at room temperature rocking and then washed again three times in PBS for 5 min. Coverslips were mounted and slides kept at 4 °C until being imaged.

Imaging using confocal microscopy

For confocal imaging, a Leica SP1 inverted confocal microscope was used. Samples were viewed and imaged using a 63x1.5 lens. Images for separate fluorophores were acquired sequentially in order to avoid bleed-through. If the intensity was to be compared between samples, laser power and photomultiplier tube settings were kept constant. Images were analysed and processed using Adobe Photoshop software. All images shown are single optical slices.

6.2.7 Protein assays

Western blots

(i) Cell protein lysates preparation

When ready for analysis, medium was carefully aspirated and 1 ml PBS added into each well of 24 or 6 well plates. Cells were detached from wells using a syringe for 24 well plates or a scraper for 6 well plates, collected into 1.5 ml tubes and centrifuged at 3000 rpm for 5 min at room temperature. The supernatant was discarded and the pellet was resuspended in 50-200 µl Laemmli's buffer. Samples were boiled for 5 min prior to be used for SDS polyacrylamide gel electrophoresis.

(ii) SDS polyacrylamide gel electrophoresis

Table 6.14 Components of resolving gels used for SDS-PAGE

%	8	10	12.5	15	17.5
30% acrylamide (ml)	2.7	3.3	4.2	5	5.8
1.5 M Tris pH 8.8 (ml)	2.5				
H ₂ O (ml)	4.8	4.2	3.3	2.5	1.7
10% APS (μl)	100				
TEMED (μl)	10				

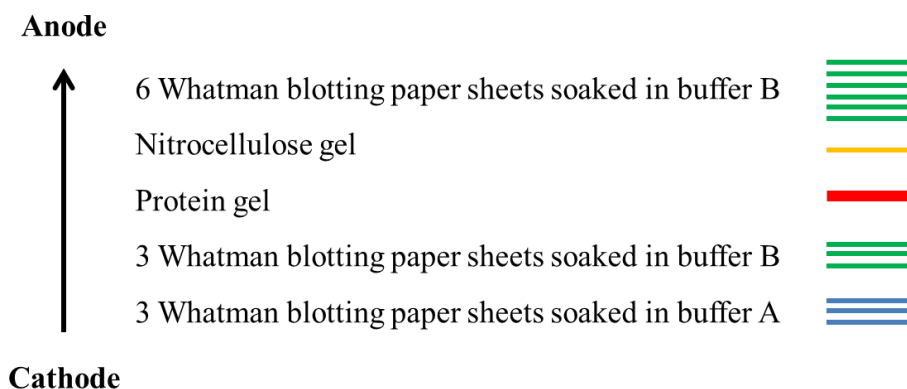
Table 6.15 Components of stacking gels used for SDS-PAGE

Component	Amount
30% acrylamide (ml)	1
1 M Tris pH 6.8 (ml)	1
H ₂ O (ml)	5
10% APS (μl)	100
TEMED (μl)	10

Resolving gels containing 8-17.5% acrylamide with a 4% acrylamide stacking gel were cast in a Bio-Rad mini-protean gel electrophoresis apparatus. Protein samples were loaded and gels run at a constant voltage of 150 V until the bromophenol blue front had migrated out of the gels.

(iii) Western blotting

Gels were transferred to nitrocellulose membranes (Schleicher and Schuell Co.) using a Bio-Rad transblot SD semi-dry transfer cell. The transfer was set up from the cathode to the anode as indicated in the diagram below.



Proteins were transferred at a constant current of 200 mA for 1hr and the nitrocellulose membrane blocked in 5% dried non-fat milk (Marvel) in PBS-T for 20 min at 37 °C on a rocker. Membranes were washed twice with PBST for 5 min at room temperature and incubated with the desired primary antibody diluted in 3 ml PBS-T at 4 °C overnight or for 1 hr at room temperature. The primary antibody was removed, membranes washed 3 times in PBS-T for 5 min and incubated with the appropriate secondary antibody diluted in 20 ml PBS-T for 45 min at room temperature. Membranes were washed 4-5 times with PBS-T for 10 min and washed once with PBS before imaging. Membranes were imaged using an Odyssey infrared imaging system (Li-Cor Biosciences).

TRAP-GFP pull-down

293T cells were seeded onto 6 well plates, transfected and when required infected with the relevant virus at an M.O.I. of 10 as described above. 48 hr post-transfection the medium on the cells was replaced with 1 ml of cold PBS with 1x PMSF (phenylmethylsulfonyl fluoride, Sigma) and plates were incubated on ice for 10 min. Cells were harvested using scrapers, collected into 1.5 ml tubes and centrifuged at 3000 rpm for 5 min at 4 °C. The supernatant was discarded and 500 µl of TRAP lysis buffer was added to the tube. The pellet was resuspended by scraping the tube against a rough surface for 10 seconds and incubated at -70 °C for at least 2 hr. Lysates were thawed on ice and centrifuged at 10000 rpm for 5 min at

4 °C. For input level analysis, a 25 µl sample was collected from the supernatant into a fresh 1.5 ml tube and 25 µl Laemmli's sample buffer was added. The remaining 450 µl supernatant were transferred to a new 1.5 ml tube containing 50 µl glutathione sepharose beads (Amersham) that had been previously washed three times by addition of 500 µl TRAP dilution buffer followed by centrifugation at 13000 rpm for 1 min. Samples (depleted of agarose-binding material) were incubated for 30 min by rotating at room temperature and then centrifuged at 10000 rpm for 5 min. The supernatant was transferred to a new tube containing TRAP sepharose beads previously washed as for the glutathione sepharose beads. Samples were incubated for 1 hr rotating at room temperature and centrifuged at 13000 rpm for 1 min. The supernatant was discarded and beads were washed three times with the addition of 1 ml TRAP dilution buffer followed by centrifugation at 13000 rpm for 1 min. 1 ml Trizol was added to the beads for bound RNA analysis by primer extension or beads were boiled in 50 µl Laemmli's buffer for protein analysis by western blot.

6.2.8 RNA assays

RNA extraction

1 ml Trizol was added to 24 or 6 well plates for RNA extraction from cell lysates or to washed GFP-TRAP beads (see above) for RNA extraction from the beads for 10 min at room temperature. 200 µl chloroform was added, mixed by inverting the tubes and left for 5 min. Samples were centrifuged at 13000 rpm for 20 min at 4 °C. The inorganic phase at the top (approximately 500 µl) was carefully transferred to a new tube, 500 µl isopropanol was added and the contents gently mixed by inverting the tubes. For RNA extraction from GFP-TRAP beads, 10 µg of carrier tRNA (Sigma) was added prior to the extraction process. Samples were centrifuged at 13000 for 20 min at 4 °C, the supernatant discarded and the pellet was washed once with the addition of 1 ml of 70% EtOH and centrifugation at 13000 for 15 min at room temperature. The supernatant was discarded and the pellet allowed to air dry before being resuspended in RNase free water. RNA samples from cell lysates were resuspended in 30 µl of RNase free water whereas RNA samples from beads were resuspended in 10 µl of RNase free water.

Primer extension assay

(i) Radiolabelling of primers

The primers used for primer extensions are listed in Table 6.1.

A mix of 1 µl PNK 10x buffer (70mM Tris-HCl, 10 mM MgCl₂, 5 mM DTT, pH 7.6), 1 µl (10 U) T4 polynucleotide kinase and 1.11 mBq ³²PγATP made up to a final volume of 9 µl with MQ-H₂O was added to 1 µl of 10 µM primer to be labelled. The mix was incubated at 37 °C for 1 hr in a water bath followed by addition of 20 µl MQ-H₂O. Labelled primers were kept at -20 °C until needed.

(ii) Transcription reaction

1 µl of RNA from cell lysates or 4 µl of RNA from GFP-TRAP beads were used for primer extension reactions and typically, two different IAV-specific primers were used per reaction in addition to a third for 5S rRNA which was included as a loading control. A mix of 0.45 µl unlabelled 5S rRNA primer (10 µM), 0.05 µl rRNA labeled 5S primer and 0.25 µl of each other primer was added to the RNA sample in a final volume of 5 µl made up with MQ-H₂O in the case of cell lysate RNA samples. Primers were annealed to RNA samples by incubation at 95 °C for 3 min followed by cooling on ice for further 3 min. Samples were pre-warmed at 50 °C for 5 min and then a transcription mix consisting of 2 µl 5x First strand Buffer (Invitrogen), 1 µl DTT (0.1 M), 0.2 µl 25 mM dNTP mix (dATP, dCTP, dGTP and dTTP), 1.55 µl of MQ-H₂O and 0.25 µl (50 U) Superscript III RT was added. Samples were incubated at 50 °C for 1 hr and denatured by adding 8 µl RNA loading buffer and incubating at 95 °C for 10 min.

(iii) Visualisation of single-strand DNA

Front and back glass plates (38 cm long) were washed with 70 % ethanol and then with 1 ml dimethyldichlorosilane before being assembled. 400 µl 10 % (w/v) APS and 40 µl TEMED was added to 50 ml 6 % polyacrylamide-urea mix (7 M Urea, 6% (w/v) acrylamide:bis-acrylamide (19:1), 1x TBE) and then poured. The gel was left to set for 1hr at room temperature.

The gel tank was prepared and each well was washed carefully using a syringe loaded with TBE before 15 µl of each sample was loaded into the gel. Gels were run at a constant

current of 35 mA for approximately 1.5 hr, placed onto Whatman blotting paper, covered with cling film and dried at 80 °C for 2-3 hr on a ThermoSavant SpeedGel SG210D gel drier. Autoradiograph film (Kodak X-Omat S; Kodak Ltd.) was placed onto the dried gel and exposed for an appropriate amount of time at -70 °C using an intensifying screen. The gels were developed using a Konica Minolta Xograph X4 SRX-101A x-ray development machine.

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